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September-October, 1959

BIOCHEMISTRY

БИОХИМИЯ

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translated from Russian

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PROBLEMS OF TECHNICAL BIOCHEMISTRY IN THE FIELD OF FOOD INDUSTRY

Among the problems raised at the XXI Congress of the Communist Party of the USSR great attention was devoted to increasing the volume of production of food products, increasing their quality, and widening their variety.

The food industry deals with raw materials of plant and animal origin, the rational treatment of which requires a deep understanding of the composition and properties of the starting materials. Soviet biochemistry has achieved substantial results in these directions in the last 20-25 years. More rational schemes for the technological processes have been worked out and introduced in many branches of the food industry: bread making, alcohol, beer brewing, molasses, wine making, tobacco, tea, and others; these have permitted a fundamental reconstruction of the industry, transforming it from a local trade into the present great industry.

Now, however, new responsibilities are given to Soviet biochemists, and for carrying them out we must raise our level of scientific investigation to new heights, taking into account the findings of biochemistry and related sciences, such as chemistry, physics, biophysics, microbiology, the physiology of nutrition, and introducing a wide variety of modern research methods: optical and luminescence analysis, isotopic methods, paper and column chromatography, electrophoresis, etc.

In many branches of the industry, especially those which deal with fermentation processes, biochemical work should be combined as a unit with microbiological investigation.

An understanding of present problems of technical biochemistry is possible only by considering the changes which have occurred in the field of the physiology of nutrition, and which compel us once more to evaluate the nutritional value of various industrial products. The old criterion of judging a food by its calorific value, based on a mechanical consideration of the organism as a machine, has long been discarded. Now, first, a qualitative estimate is made of the nutritive power of the product and this determines all the planning of the production of the food product. For example, the science of nutrition in term of proteins has changed completely from determining their total amount to determining the essential amino acids. This requires an extensive study of the amino acid composition of the original raw material and the food product, since their differences may be great in respect to these essential acids. Also, there is the important question of the more rational use of products with valuable amino acid content, such as milk protein.

There has also been considerable change in the idea of the comparative value of different fats. The widespread idea that the best fats were those of animals (first of all of milk) has become very doubtful. Actually, animal fats as compared to plant fats are poor in essential fatty acids, linoleic and linolenic, which the human organism cannot synthesize, although it needs them. On the other hand, animal fats contain relatively more cholesterol. If such a combination is not the direct reason for sclerotic changes in the walls of the blood vessels, at any rate it predisposes them to these changes. Therefore there is good reason to increase the relative amount of plant fats in foods. Until recently it was considered that vitamins protect the organism from avitaminosis, for which rather small amounts are needed in the food; but now it has been established that increasing the vitamin content to the physiological optimum norm leads to increased resistance by the organism against all types of unfavorable influence, including infections. The mineral content of food products, especially the microelement content, has great significance for the value of the food, but this question has not been sufficiently studied in the USSR. In this respect we may expect great help from the departments of inorganic and analytical chemistry of the universities, which can carry out studies in cooperation with different branches of industry, a very desirable condition.

In connection with the changes in the scientific basis of nutritional requirements, and also with the increase in public health and the sharp increase in the production of food products in the next seven years, we must make fundamental changes in a number of processes. This cannot be done merely by a rationalizer of the work. The problems can be solved only by a deep study of the theoretical basis of production and by furnishing new methods of technology. In spite of the various food sources and methods of treating them, the main directions of investigation in technical biochemistry can be grouped around the following questions.

1) Questions of the biochemical study of the technical quality of the raw resources, including biochemical control of selection, agricultural techniques, and also conditions for maintaining and feeding domestic animals.

2) Questions of maintaining the quality of the raw materials during treatment and keeping.

3) Questions of rational technology during technological treatment of the raw materials.

4) Increasing the nutritive and flavor values of industrial products, including enriching them with proteins and vitamins, and strengthening aroma, flavor, etc.

5) Questions of rational use of wastes.

6) Working out new, rapid methods of technological control.

Problems of the quality of the original raw materials concern all branches of the food industry. The solution of these problems lies in the work of the breeder and the agricultural technician, if we speak of plant materials, and is a problem for the animal technician for materials of animal origin. However, this work can be carried out successfully only with the direct participation of the biochemist who constantly watches for changes in the technical quality of the material. The breeder and the animal technician devote most of their attention to the quantitative side and do not always consider the needs of the food industry; therefore the quality of the material worsens. This can be especially illustrated by the example of our wheat. Such high quality varieties as "Ukrainka and Kooperatorka" have now disappeared from the sowing. Their place has been taken by wheat with very poor bread making quality, low grade and easily attacked by proteolytic enzymes of gluten. Besides, even if it is easy to remove such deficiencies in the baking industry, deficiencies which occur in the carbohydrate complexes of the flour, still the deficiencies in the protein complexes, on which the value of the bread chiefly depends, cannot be corrected by technology and can cause harm to the consumer. Workers in the canning industry well know the example when a new type of tomato was introduced only because of its apparent high yield in the harvest, and in the actual factory treatment had only an additional quantity of water.

Thus, it is quite evident that in the selection process it is always necessary to consider the biochemical indications of quality, as otherwise many years of work by the selector may be in vain. It is therefore necessary to set up a network of biochemical laboratories at the selecting stations.

The quality of the raw plant materials should be the most important criterion not only in the work of selection, but also in judging the different measures of agricultural technology. Defects here lead to very unfavorable consequences. A graphic example which illustrates this point is given in the case of incorrect irrigation of a sugar best plantation. This sometimes produces an increased harvest of the beets, but their sugar content is lowered proportionally. Hence there is here no increase in yield of sugar per hectare, but there is merely an increase in load during transport to the sugar factory. Another example: the harvest of tea leaves per hectare can easily be increased by application of nitrogenous fertilizers. However, the leaves are then so enriched in proteins that a high quality black tea cannot be obtained, since most of the tannins then go into the insoluble state during the fermentation process.

It follows from all this that there must be extensive research on the effects of a number of agricultural procedures (especially irrigation and fertilizing) on the quality of a number of products in various areas of the Soviet Union. This applies most of all to growth of grains and production of oils, for which the important criteria of quality should be the content of proteins and fats, and also to vegetables and fruits, for which the criterion is their richness in carbohydrates, vitamins, tannins and other compounds which are important in a nutritional sense.

Since gathering the harvest involves a definite short period and the work of the preparative industries if possible should not have a seasonal character, a very important problem in the food industry is to work out a rational regime for keeping agricultural products. This applies to keeping grains, and particularly to preserving juicy raw materials. Since the chief method of preventing spontaneous heating in moist grains is to dry them,

there has long been the important problem in grain biochemistry of working out and intensifying the methods of drying the grain mass. This involves working out the biochemical basis of the grain drying process using the principle of stepwise drying in which the temperature of the drying agent is raised as the grain is dehydrated. It is particularly necessary to note the importance of working out the physiological and biochemical basis for drying the seed grains, especially corn. Most attention should be devoted to finding new methods in the technology of keeping grains. Here it is most necessary to indicate the problem of the use of short wave irradiation for preserving the grain from spontaneous heating and the use of different chemical compounds and antibiotics. The preservation of the quality of oil-bearing seeds when they are kept is a serious problem.

New problems are presented to the biochemist in the field of keeping juicy raw materials. In the years which preceded the first Five Year Plan there was discussion in the Scientific Research Council of the sugar industry as to whether to plan keeping the beets in the factory for 100 days or whether the period of keeping should only be 80 days. Since in the voting on this question the vote was evenly divided, the norm was set at 90 days. To day this seems ridiculous.

Only a prolonged period of keeping the beets, based on a deep investigation, could solve the problem in the first Five Year Plan by a sharp rise in production of sugar without further capital investment in the construction of new factories in the older beet growing regions. At the present time the sugar industry has almost doubled the period of keeping the beets compared to the old standard. However, the Seven Year Plan presents to scientific workers great new problems whose solution should eliminate seasons in sugar production.

We must mention the present severe lag in production of fruits for the needs of the population. Actually, the planting of new orchards is the radical solution of this problem, but it requires great capital outlay which can be realized only after a number of years. Therefore it is of first importance to work out methods for the rational preservation of fruits and barries which will about double the availability of these valuable products for the people. How can this be done ? Actually, it is easy to keep fruit by stoping the completion of biochemical processes in them at low temperature, for example, by keeping in a refrigerator. However, this is hardly rational from the economic point of view. We must also find agents which will slow these biochemical processes at ordinary temperatures. The solution of this problem is possible only by the fullest use of the natural properties of fruits and vegetables which resist microorganisms and functional destruction. Important in this respect is the study of the biochemical nature of immunity in potatoes, vegetables and fruits. An important part should be working out of those methods of sterilization, drying, and freezing of fruits and vegetables which would assure the possibility of obtaining the effect with more positiveness and with shorter periods of action. We must very soon work out and introduce into production new methods for keeping potatoes, vegetables and fruits based on the use of radioactive irradiation. The use of gamma radiation in this way offers great possibilities. Experiments carried out on the irradiation of potatoes in the A. N. Bakh Institute of Biochemistry showed the possibility of keeping them without loss for two years. In this connection we must have a series of biochemical studies directed to explaining the effects of ionizing radiation on metabolism of the storage organs of plants. We must quickly complete the construction of the experimental industrial equipment to allow the Institute of Nutrition of the Academy of Medical Sciences of the USSR to finish its wide physiological study of the problems of possible use of irradiated potatoes for both technical and food purposes, and on the basis of this work to reach a technical solution on a suitable scale. With reference to new technological principles in the food industry there are tremendous opportunities. This can be understood from a consideration of the variety of products with which we deal. It is scarcely possible to consider all this extensive material in this paper. We can only set up the general scientific basis for the rationalization of most of the technical processes of the food industry. In principle, these processes are the following: as a result of some kind of action the living cell is broken down and passes into the production of a plant or animal raw material. However, enzymes included in these materials remain active, and they also cause chemical changes in the production mixture and transform the raw material into the final product, making it easier to transport and more stable in storage; they increase the nutritive value and add flavor and taste for the consumer. Sometimes, in addition to the enzymatic processes there are changes which depend on the introduction of special microorganisms into the products. Illustrations of this are those processes which occur in rising dough before it is put in the oven, in fermenting beer and the must of wine, in tea leaves rolled and crushed, in fermenting tobacco, and also in salt fish, preserved meat, and ripened cheese. The technological problem in all these products is to consider the quality of the starting material, and by the use of some external action (temperature, moisture, acidity, etc.) to guarantee the proper course of the desired process, strengthening some reactions and weakening others in the reaction mixture, and thus improving the quality of the final product.

Even to day food technology offers a number of examples which show that only a deep knowledge of the properties of such enzymes as α- and β-amylases, various proteases, oxidizing enzymes, etc. can permit rationalization of the technical processes and control of the situation even when the raw material does not come up to the standards of production.

Thus, these problems which are presented to the food industry insistently demand from biochemists a broadening in the number of cases of application of our theoretical knowledge to practice. However, along with the rationalization of production and the increase in quality of the resulting products, we should devote serious attention to an increase in the number of products which do not require great capital outlay. This can be illustrated by the examples of an industry which is comparatively small but which produces a very important dietetic product, the tea industry. The tannins in tea have a favorable effect on the blood vessels, strengthening them. However, tea production among us falls behind the demands of the people. In addition, our tea is rather poor in tannins and the amount of these is considerably decreased in the production (fermentation) of black tea. The solution of this problem only by establishing new tea plantations is scarcely reasonable. It is important to rationalize the technology, or more accurately, to find a new technological method of tea production. We must improve the production of the so-called crystalline tea from the wastes obtained from the clippings and shed parts of the tea bush. Perhaps we can increase the production of green tea, using special plantations whose harvests can be increased by nitrogenous fertilizers, as is done in Japan, etc.

In the present Seven Year Plan we must have a sixfold increase in vitamin production in order to satisfy fully the needs of medical institutions and assure wide fortification of food products directly by vitamins and also to supply vitamins for the various branches of animal industry (swine and poultry raising) in order to increase productivity. The production of vitamins now takes place most economically by the synthetic method, but for some vitamins the natural sources have not lost their value, in view of the absence or complexity of synthetic methods for obtaining them. It is also important that in a number of cases the natural products contain a combination of vitamins which is physiologically more valuable than is the separate administration of the components, as in the hedge rose, black currant (vitamins C and P), or in yeast (vitamins of the B group), so that the use of such sources should be widened. Among the directions in which biochemical investigations of the vitamin industry should be carried out are the attempts to widen the variety and increase the quality of the plant sources (for getting a high yield of carotene, flavinoids, and especially, rutin) and to perfect the conditions for culturing the microorganisms which are sources for a number of vitamins. Here belong studies of conditions for culturing yeast to get a maximum yield of ergosterol. Investigations designed to show the path of biosynthesis of vitamins and their formation from precursors are also very promising. It is possible that the introduction of riboflavin precursors into the medium will intensify the process of formation of this substance in cultures of microorganisms. As part of this work we find the increase in accumulation of vitamin B_{12} by directing the fermentation of propionic bacteria.

A major problem before biochemists in the Seven Year Plan is the great increase in milk production. This problem is the more serious because at the present time views are changing as to the nutritive value of the different components of this product. Even recently the particular value of milk was believed to lie in its fats, and the nutritive value in using its proteins, vitamins, and salts was not fully realized. By 1965 there should be produced in the Soviet Union about a million tons of milk fat. Such a quantity has not been produced, and judging by the tempo of growth, will not be produced in any country in the world. For the production of such an amount of fat about 20 million tons of milk per year would be required. This raises the problem of the use of the defatted milk. The present methods for drying defatted milk are unsatisfactory. Here new methods must be worked out which will permit the use for nutritive purposes of the proteins and vitamins of defatted milk. Also, the use of whole milk products should be sharply increased, which is related in a number of cases to their lack of stability when kept and the difficulty of their transportation. The treatment of fresh milk, in its bacteriocidal stage in the short period after milking, when the bacteria which enter milk have not only not developed, but have been destroyed, would guarantee production of a high quality raw material, sour cream, curds, and other products. Therefore it is necessary to turn our investigation to the study of the chemical nature of the bacteriocidal substances of milk and to seek methods for their use to prolong the bacteriocidal stage in order to apply it directly in milk treatment. There is a great field for biochemical work in the study of processes which go on in the preparation of sour milk products, and also in the ripening of cheese. Here empiricism still rules, and life insistently demands the development of new methods and the preparation of new types of products rich in proteins and vitamins.

In concluding this section, we must mention the production of enzymatic products. They are useful in a wide variety of food industries; however, their production will be practical only in the coming Seven Year Plan. As already mentioned above, an understanding of the properties and behavior of enzymes will chiefly permit us to set up problems of improving their action in treating natural raw materials, and in this direction Soviet biochemists have achieved considerable progress. The problem here is mainly the improvement of the action of those enzymes which are present in raw materials which are being treated. New and very great possibiliteis have been opened up in recent years in connection with the preparation of enzymes in concentrated form from cheap sources - must fungi and bacteria which can accumulate large quantitites of enzymes and grow on the wastes of the food industry. The advantages of the use of enzymes is very great, as is shown by the rapid growth in the demand for them and the quick development of special industries for their production, especially in such countries as the USA and Japan. The use of enzymes permits intensification of production processes, lower the demand for valuable nutritive materials, increases the quality of production, and supplies new forms of products for technology, food, and medical uses. As an illustration we can give the results of experiments with yeast amylase carried out by the baking industry in Moscow. The introduction of 20 grams of the preparation per ton of white flour greatly improved the quality of the bread: sugar content, volume, porosity, taste, odor of the crumbs, and color of the crust. Positive results of using enzymes were obtained in the alcohol, beer, wine, and a number of other branches of the industry.

The creation of an enzyme industry is a new step in the improvement of biochemical processes which take place in the treatment of food materials, but answers must be obtained to the complexities which occur in the problems which arise. They include the selection and choice of strains of highly productive microorganisms, working out conditions for culturing them, growing, stabilizing, and standardizing the active preparations, etc. Therefore we should be ready to strengthen considerably the microbiological, biochemical, and technological studies.

An important problem before the food industry is the rationalization of the use of food wastes. In the hydrolytic and alcohol industries there is the problem of greatly increasing the production of nutritional yeast on alkali sulfites and residues from distillation. However, we should also consider that the wastes can be used for obtaining still more valuable nutritional products. Thus, for example, the culturing of actinomycetes is very effective, and gives a mass of mycelia with a high content of vitamin B_{12} and antibiotics together, a mixture mos suitable for use in livestock raising. According to our information, such production already exists in the USA and Western Germany. We have inexhaustible sources of various valuable organic products in the wastes of the sugar industry, the press cake and molasses, which have still not been used rationally. The wide use of nutritional molasses for alcohol production will gradually be reduced in view of the wider introduction of cheap chemical methods for obtaining technical alcohol. This raises the question of diverting the use of molasses into other channels so as to obtain valuable nutritional and food products. The problem of the rational use of the press cake is especially the problem of using the very valuable nutritional proteins which it contains; this still awaits solution. There are still a large number of unused waste products in the fish industry, and among them are wastes which can be valuable sources of fats, foods and technical products, vitamins, hormones and enzyme preparations, protein hydrolyzates, and various amino acids.

Finally, there is a much smaller amount of wastes from such branches of the industry as tea or viticulture, but these wastes, such as grape residues and seeds, can usually be used to obtain organic acids, tannins and dyestuffs, etc. The preparation of such products, and also of various preparations of vitamins and proteins useful in nutrition, etc. is being applied more and more in connection with the increasing use of refined food products (white bread, macaroni, sugar, and others). These products contain in themselves little or no vitamins and other requirements for nutrition, flavor or good diet. This raises the problem of increasing in every way the nutritive and flavor values of food products by adding supplementary substances to them, enriching them with proteins, vitamins, aromatic substances, introducing flavoring compounds, definite mineral complexes, etc. This presents to a number of branches of the food industry the problem of working out and introducing into production rational methods for such enrichment of foods and this is possible only by wide use in the work of methods of scientific analysis and strict biochemical control.

The wide introduction of biochemical control into a number of branches of the food industry is generally the greatest problem which faces biochemists. Actually, it would be difficult today to carry out the work of such highly developed industries as that of sugar, for example, without constant control in all stages of the plant. At the present time, biochemical control exists in all tea factories in which, even in recent times, all the operations

of wilting the tea leaves, twisting them, fermentation, and drying were literally based only on the experience and intuition of the technologist. Today, each of these production operations is controlled by objective chemical methods which greatly improve the quality of the final product. Determination of enzyme activity and susceptibility of the substrate in different batches of flour often permits control of kneading in bakeries, carrying out the process not by chance, empirically, but on fully reasonable grounds. However, these attempts are still far from being used widely either directly in bread making or before delivery of the flour to the bakery, in the elevators and mills where the grains are classified. Usually biochemical control is a rare guest in food production, while its wide use would greatly improve the production and raise the quality of the final product. The present level of biochemical analysis allows us to follow the course of different processes with great accuracy, and also to determine very axactly the formation of various products in the complex production mixture. Unfortunately, those control methods which in many cases are used in the factories are usually still very rough and are a long way from the possibility of giving results to which our laboratory workers are accustomed. It is very important to introduce widely automatic methods of continuous control.

The problems which the Seven Year Plan presents to the domestic food industry are very great. We can see that their solution is possible only by taking into account all the results of biochemistry in the field of theory, and also the new scientific progress which the nation expects from its scientists. There is every basis to hope that Soviet biochemists who have already carried out much work of a practical nature will succeed in the solution of these problems.

A. I. Oparin.

SOME PROPERTIES OF COMPLEX COMPOUNDS OF PROCOLLAGEN AND BACTERIAL NUCLEIC ACIDS

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In recent years there have been detailed studies of the pathogenesis of the attack on connective tissue observed in the so-called "collagen disease". It is widely held that in the development of this condition there is a special role of an allergy to the desoxyribonucleic acid (DNA) of microbial or other origin [1]. Direct evidence of the ability of bacterial and animal nucleic acids (NA) to produce an allergy has been presented by Bardwill et al. [2] and by Swineford and Holman [3].

It is known that DNA has little or no antigenic activity [4]. There is great theoretical and practical interest in discovering the mechanism which leads to the appearance of allergenic properties in DNA. The work of Mayer and Sammit [5, 6] is of interest from this point of view. These authors found by creating artificial antigens that procollagen used as a protein-carrier sharply increased the ability of some substances (picryl chloride, old tuberculin) to produce allergies. In the opinion of these authors, the allergenic properties of various substances with respect to the skin depend on their ability to combine with procollagen.

In a study of various bacterial factors which could act on collagen, we found that procollagen can react actively with bacterial DNA [7]. We used this property of procollagen to obtain preparations of bacterial DNA.

We set ourselves the problem of using the DNA preparation to study the properties of the compound of procollagen with DNA: its character (complexity), quantitative ratio of the components, effect of various factors on their ability to react with each other, and, finally, the effect of DNA on some specific properties of procollagen, especially its stability toward proteases. The interest in the latter question comes from the absence of any clear idea of the mechanism of splitting of collagen in different pathologic states. A study of the properties and conditions of formation of the compound of procollagen and bacterial nuclei acids, from our point of view, can be of some value in explaining the biochemical aspects of collagen disease.

EXPERIMENTAL PART

Analytical Methods

DNA was determined by the diphenyl amine method of Dische, hydroxyproline by the method of Neuman and Logan [8]; the absorption spectra of NA and compounds of NA with procollagen were measured on an SF-4 spectrophotometer using 1M NaCl as solvent; phosphorus was determined according to Fiske-Subbarow; nitrogen by the micro-Kjeldahl method. The native state of the NA preparations was judged by spectrophotometric tests based on the ability of native preparations of NA to increase its extinction at 260 mμ in an alkaline medium [9].

Materials Used

Procollagen

This was obtained from rabbit skin by the method described by Tustanovskii [10]. We used a preparation dissolved in 0.01 N CH_3COOH. The absence of admixed NA in the preparation was confirmed spectrophotometrically and by the diphenyl amine test of Dische.

Microbe Strains *

The NA preparations were isolated from strains of Shigella dysenteriae Sonnae No. 9529 and Staphylococcus pyogenes "Lossman". We used 18-and 24-hour cultures grown on solid nutritive media.

Crystalline Trypsin **

Crystalline DNA-ase***

NA Preparation

This was obtained by the following method, which we worked out: the 18 - hour culture was washed with 0.1 M solution of sodium citrate nutrient medium. The cells were separated by centrifuging, washed with the same solution and ground in a glass homogenizer for 30 min in the presence of dry sodium citrate (1 g to 7.5 g of moist bacteria) and powdered glass (2 g per 1 g bacteria). The resulting mass was treated with ice distilled water calculated at 30 mg of citrate per ml of water. Extraction was carried out for 48 hours at 0°. The remaining residue of cell fragments and glass was removed by centrifuging and the supernatant liquid was treated with 2-3 volumes of procollagen solution. The mixture remained in the cold for 2 hours and then the precipitate was separated by centrifuging and dissolved in a 6% solution of sodium para-aminosalicylate (PASA, the usual pharmaceutical preparation), under the influence of which, according to Kirby [11] there is splitting of the compound of protein with DNA. The liberated protein was removed by repeated treatment with a mixture of chloroform and isoamyl alcohol (9:1) by the method of Sevag. The liquid from which the protein had thus been removed was treated with 1.5-2 volumes of 96% ethyl alcohol. The resulting fibers of NA were removed mechanically by winding on a fine glass rod. The preparation was dissolves in 0.2 M Na Cl and precipitated by alcohol and dried in a vacuum desiccator.

In the preparation obtained from the dysentery bacteria we found 6.6% phosphorus and 13.3% nitrogen, and in the preparation from the "Lossman" strain, 8% phosphorus and 14.26% nitrogen. We found in these preparations 70 and 81% respectively of DNA by Dische method. The amount of phosphorus found indicates that in the preparation from the dysentery carriers there was about 68% NA, and in the preparation from the "Lossman" strain, 83%. Comparison of these results with the figures obtained in the determination of DNA by the Dische method allows us to conclude that ribonucleic acid is either completely or almost completely absent. The native character of the resulting preparations is confirmed by the increased extinction at 260 mμ when alkali is added to the NA solutions. Thus, the extinction of the solution obtained from strain No. 9529 is increased by 26%, and from the "Lossman" strain, by 24.2 %.

After 24-hour hydrolysis with 6 N HCl we found the following amino acids by paper chromatography in the composition of the NA obtained from these strains: histidine, arginine, glycine, hydroxyproline, glutaminic acid, alanine, threonine, proline, (we placed the hydrolyzate from 1.5 mg of preparation on the chromatogram). The group of amino acids which we found in the NA preparations indicates that they do not contain a random group of proteins, but rather polypeptides or amino acids which are firmly bound to the DNA molecule.

We should remark that proteins, polypeptides, or separate amino acids are constant admixtures in preparations of native NA obtained by different methods [11-13]. Frick observed amino acids in carefully purified preparations of DNA obtained from calf thymus by the method of Gulland. Moreover, Keil' and Grubeshova [14] found that complete removal from RNA of its protein admixtures resulted in its depolymerization.

COURSE OF THE EXPERIMENTS

In order to show the quantitative ratio between procollagen and DNA in the compounds, we ran experiments with NA preparations isolated from the "Lossman" strain.

* The dysentery-producing strains were obtained from the museum of the department of microbiology at the Rostov-on-Don Medical Institute, and the "Lossman" strain from the L. A. Tarasevich Control Institute for Vaccines and Sera, Moscow.

** The preparation was kindly supplied by the Laboratory of Protein Chemistry of the Institute of Biological and Medical Chemistry, Academy of Medical Sciences, USSR, Moscow.

*** This was prepared in the "Soyuzreaktiv" Combine.

To 0.5 ml of 1/15 M phosphate buffer (pH 7.2) was added an equal amount of NA (100-300 μg) in 0.3 ml of 0.2 M sodium chloride solution and 0.2 ml of procollagen solution (1.5 mg/ml). The final pH value of the mixture was 7.05. The sample remained in the cold for 2 hours, after which the precipitate was separated by centrifuging. DNA was determined in the precipitate and in the supernant liquid (as shown above, the preparations used consisted chiefly of DNA).

In the study of the effect of various factors on the ability of DNA and procollagen to react with each other, we used DNA preparations which were first denatured by heating for 20 min at 94°, as recommended by Schak [15] or had been submitted to the action of desoxyribonuclease (0.5 mg/ml in the presence of 0.005 M $MgCl_2$ at pH 7.2) for an hour. Also, we used procollagen solutions heated for a moment to 100°.

In some experiments we studied the reaction of procollagen with NA found in the extracts from the bacteria ground with glass. The extraction was carried out with 1/15 M phosphate buffer (pH 7.2) for 4 hours at 0°. In these experiments we determined the total amount of DNA in the extract and also the DNA which combined with procollagen under these conditions.

The stability of the procollagen combined with DNA toward the action of proteases was studied by submitting the complex to transformation by crystalline trypsin. The procollagen was combined with different amounts of NA preparations under the same conditions as in the experiments to show the amounts of reacting DNA and procollagen. The compounds of procollagen and DNA were centrifuged, mixed in 0.01 N CH_3COOH and treated with an equal volume of 1/15 M Na_2HPO_4 to which was added crystalline trypsin (2 mg/ml), all cooled to 0°.

As a control we used procollagen (in equal amounts) precipitated at a neutral pH. This pH was obtained by adding two volumes of phosphate buffer (pH 7.2) to the starting solution of procollagen. The procollagen was separated by centrifuging, dissolved in 0.01 N CH_3COOH and trypsin in a solution of Na_2HPO_4 was added to it (control A, Table 2). A second control, control B, (Table 2), consisted of a compound of procollagen with DNA, incubated under identical conditions but without addition of trypsin.

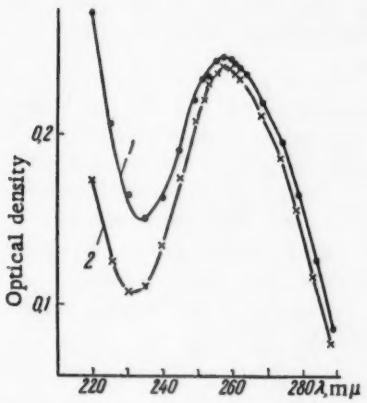

Fig. 1. NA absorption spectrum for the free state and combined with procollagen. NA isolated from the strain Staphylococcus pyogenes "Lossman": 1) NA combined with procollagen; 2) NA in the free state. Concentrations the same in both cases.

Incubation of the controls and experimental samples was carried out for 18 hours at 28-30°, since denaturation of skin procollagen from mammals begins at 36° [16]. The presence of trypsin action was judged by the increase in hydroxyproline in the supernatant liquid. At the end of the incubation the part of the complex which had not been split was separated by centrifuging, and in part of the supernatant liquid after 24-hour hydrolysis at 105° with 6 NHCl we determined the hydroxyproline. The addition of an equal volume of 20% TCA to the supernatant liquid did not change the amount of hydroxyproline which it contained.

RESULTS AND DISCUSSION

The compound of procollagen and DNA differs in its physicochemical properties from native procollagen. Its formation occurs slowly when procollagen is added to the solution which contains DNA. The compound precipitates as a finely dispersed solid, insoluble in 0.01 N CH_3COOH and soluble in 1 M NaCl. Under the same conditions (ionic composition, value of ionic strength, and pH of the solution) procollagen precipitates much later in the absence of DNA, has a characteristic fibrous form, dissolves in 0.01 N CH_3COOH, and is insoluble in 1 M NaCl. Especially significant is the fact that the compound of procollagen with DNA acquires the ability to dissolve in 1 M NaCl. It is well known that native procollagen is salted out of solution by the addition of 1.35-5% NaCl to it.

In the reaction with DNA the stability of procollagen to the action of proteases is changed. We have established this fact and described it below.

DNA in combining with procollagen loses the ability to dissolve in 0.2 M NaCl. The absorption spectrum in the ultraviolet for the compound of procollagen and DNA differs from the absorption spectrum of pure NA at the same NA concentration in solution (Fig. 1).

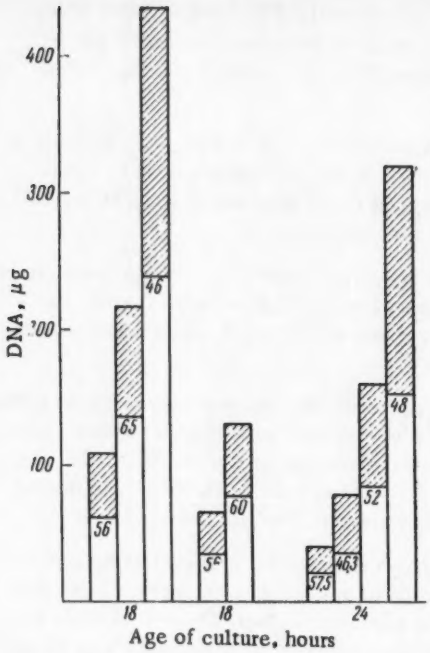

Fig. 2. Amount of DNA combined with procollagen DNA extracted from cultures of Staphylococcus pyogenes "Lossman". Shaded part of column – free DNA, unshaded part – DNA bound with procollagen. Figures in columns – percent of total amount of DNA in the extract.

Here we should mention that Manoilov et al. [17] established the formation of nucleoprotein complexes by the change in ultraviolet absorption spectra. Thus, the considerable change in properties of procollagen and DNA observed with very short contact at low temperature, and at neutral pH are similar. From our point of view the only reason for these changes is the formation between these components of a complex type of compound.

Study of the quantitative ratio of DNA and procollagen in the complex shows that the amount of DNA which combines with a definite amount of procollagen depends on the state of the DNA molecule and the state of the procollagen molecule. In the experiments carried out with DNA isolated from the "Lossman" strain we found that 22.6% DNA was combined with procollagen. In the conditions in which the preparation was denatured by heat (breaking hydrogen bonds [18]) this amount increase to 33%. The action of DNA-ase decreased the amount of DNA which could react with procollagen to 2.9%. On the other hand, momentary heating of procollagen to 100° completely prevented its combination with DNA.

The formation of complex compounds of DNA and procollagen occurs when the same amount of procollagen (1.5 mg) is added to an extract of destroyed bacterial cells obtained with a phosphate buffer. In these experiments, independent of the concentration of DNA in the extract there is combination with procollagen of only a definite part of it (about 50% DNA) (Fig. 2).

This findings permits us to assume that the DNA in the extract is nonhomogeneous in its degree of polymerization, since the ability to combine with procollagen is possessed only by highly polymerized DNA. Such an assumption is based on the data obtained concerning the decreased ability to combine with procollagen under the influence of DNA-ase. This assumption has been confirmed: it has been shown that an amount of DNA precipitated by adding an equal volume of normal HCl to the extract (this is known to precipitate polymerized DNA [9]) agrees with the amount of DNA which can be removed from the extract by a great excess of procollagen, three to four volumes (Table 1).

TABLE 1

Depolymerization of DNA from Different Sources according to the Results of Precipitation by 0.5 N HCl and Excess Procollagen (in percent of total amount in the extract)

Source of NA	Na not precipitated by 5N HCl	NA not precipitated by excess procollagen
Extract of 18 hour "Lossman" culture	8,3	8,0
The same	8,3	10,0
Extract of 72 hour "Lossman" culture	16,0	16,0

This viewpoint is confirmed by the fact that such measures as addition of a large amount of citrate and bacteria (factors which hinder the action of DNA-ase) beginning at the moment of destruction permit the precipitation by procollagen from the extract of considerably more DNA, up to 90% (Table 1), than in the cases where the destruction and extraction are carried out in the absence of citrate, about 50% (Fig. 2).

This ability of procollagen to form an insoluble complex with polymerized DNA molecules, which is precipitated by acids, and not to react with degradation products of DNA allows us to recommend procollagen as a reagent which can separate these products, the more so that from the compound with procollagen it is easy to obtain DNA again in the polymerized state.

At the present time, acids (hydrochloric, acetic) are often used for this purpose. This acidification precipitates the NA polymer [9], but it must be remembered that acid in these concentrations can itself be a reason for denaturing changes of DNA [18]. The ability of procollagen to form complex compounds with DNA as used in our method gives preparations of native bacterial NA.

In various method which have been worked out for obtaining such preparations from living objects ethyl alcohol is used in the early stages to precipitate nucleoprotein fibers which are transferred mechanically into 1 or 2 M NaCl. In these solutions the nucleoprotein dissociates into NA and protein. The use of such methods for obtaining bacterial NA is not always possible, since sometimes the nucleoproteins precipitated by alcohol do not dissociate further in solutions of sodium chloride or other salt solutions [19]. We have also encountered a similar effect in trying to obtain a preparation of NA thus from the strain of Shigella dysenteriae Sonnae No. 9529.

This pecualirity of bacterial nucleoproteins makes it necessary to work out a special method for obtaining bacterial NA. Thus, Phillips et al. [13] exclude altogether the procedure of precipitating by alcohol in the early stages, although this results in getting a rather impure product. Jones [20] precipitates NA at this stage by a special reagent, Cetavlon. For the precipitation of NA we have used procollagen obtained from rabbit skin. The method of obtaining this has been worked out in detail by Orekhovich and Tustanovskii [21] and can be used very simply and with success in any laboratory.

The preparations obtained by this method do not differ appreciably in purity from the native preparations obtained by other methods used for isolating bacterial NA, such as the method of Jones [20]. However, this method has some peculiarities. By its use, especially with some materials, we obtain a preparation in which ribonucleic acid is either absent or almost absent, and it is not necessary to use such procedures as treating with RNA-ase or Norit [22]. The absence of RNA in the resulting preparations is perhaps connected with the presence of active RNA-ase in the strains which we used.

Action of Crystalline Trypsin on the Complex of Procollagen with DNA

As a result of the formation of the complex compound between procollagen and DNA various physicochemical properties of the protein are changed, as we have described above. In this section we give results of the change in stability of these complex compounds toward the action of trypsin. A study of the factors which lead to a decreased resistance of procollagen to proteolytic enzymes is of great interest for an understanding of the mechanism of splitting of collagen in some pathological states.

As is known, the stability of procollagen to the action of most known proteases depends on its amino acid composition and structural peculiarities [23]. The fact of a change in the stability of this protein to trypsin when it forms a complex with DNA is in itself evidence that as a result of such a reaction there is a rather deep change in the protein part of the complex.

As the enzyme which acts on the complez we chose trypsin. Here we considered that this enzyme has many common features (optimum action, relation to inhibitors) with necrosine, the protease isolated from the exudate of inflammations and purified by Gorkin and Kondrashova [24]. The conditions for running the experiments were described above, and the results are given in Table 2.

For the results given in Table 2 it is quite evident that the formation of a complex compound of procollagen and NA is accompanied by a decrease in the resistance of procollagen to the action of trypsin. These results are interesting when compared with those obtained by Forcher and Cecil [25]. These authors found that the breakdown of collagenous tissue in inflamation occurred only after the accumulation of a large amount of DNA which was fixed by the components of the tissue.

We can assume that the formation of the complex of DNA with procollagen occurs in this inflammation. This perhaps also lowers the resistance of the latter to the action of the tissue proteases. Here it is proper to mention that an accumulation of considerable DNA in the composition of connective tissue is often found in some collagen diseases [26].

In conclusion we should remark that the pathogenesis of collagen disease is connected with the development of allergy to DNA. It will require detailed experimental study to explain the role of the complex compound of procollagen and DNA in the development of the conditions characteristic for some "collagen diseases". Preliminary results which we have obtained indicate the possibility that this compound causes allergy to DNA in experiments with rabbits.

TABLE 2

Action of Crystalline Trypsin on the Complex of Procollagen with DNA from Different Sources

Experimental conditions	DNA combined with 1 mg procollagen, μg	Hydroxyproline found in supernatant liquid, % of total amt. in procollagen sample.
NA preparation obtained from Shigella dysenteriae Sonnae No. 9529		
Incubation at 30°:		
Experimental	120	33,4
Control A (native procollagen and trypsin)		6,3
NA preparation obtained from Staphylococcus pyogenes "Lossman"		
Incubation at 28°:		
Experimental	155	22,9
Control A (native procollagen and trypsin)		.7,4
Control B (complex of procollagen and DNA without trypsin)	155	0
Ectract of "Lossman" strain		
Incubation at 30°		
Experimental	155	34,2
Control A (Native procollagen and trypsin)	—	7,64

SUMMARY

We have established the fact of formation of a complex compound of procollagen with bacterial DNA . The ability to for this complex depends on the condition of the DNA molecule and the condition of the procollagen molecule.

We have worked out a simple method for obtaining bacterial NA by precipitating it with procollagen. The procollagen which enters the complex with DNA is much more strongly altered by trypsin than is native procollagen.

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* See English translation.

THE SURFACE LAYER OF DIPHTHERIA BACTERIA CELLS AND ITS TOXIC LIPIDS

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On the basis of the investigations of Stacey [1-3], Sponner [4], and Dubos [5] the idea has grown up that the surface layer of Gram-positive bacteria consists of a complex of proteins and magnesium salts of ribonucleic acids which cause their Gram-positive properties.

Since we [6] have observed in combination with such proteins 22% of lipids, we have reason to assume that the surface layer of diphtheria microbe cells is evidently not a simple nucleoprotein, but a liponucleoprotein. In order to test this idea, we have undertaken the separation of the surface layer of diphtheria bacteria cells from the main "skeleton" of the cells and a study of the action of the lipids of this layer on the organism of animals. The work of Stacey [1, 2] is basic to this; he has succeeded in separating from the surface layer of the cells of a number of bacteria the main part of the Gram-positive complex and has obtained the Gram-negative "skeleton" which retains the form of the organism.

EXPERIMENTAL PART

Separation of the Bacterial Surface Layer

In order to separate the surface layer we shook 100 g of diphtheria bacteria, strain PW-8, dried at 37°, for 6 hours with 1 liter of 2% solution of sodium taurocholate. This extraction was continued to full loss of Gram-positive properties by the bacteria. Each series of extracts was separated by centrifuging and the bacterial residue was treated with a fresh solution of sodium taurocholate. The first filtrate was separated into three parts. In one of these (1), the protein was precipitated by alcohol, in another (2), acidification by acetic acid was carried out. The third portion was dialyzed in a collodion bag against distilled water at 3°. The dialyzation water was evaporated to dryness and extracted with ether. The residue after evaporation of the ether was designated E-1.

The proteins precipitated by alcohol (P_1) and by the action of acetic acid (P_2) were washed with alcohol, then with ether with centrifuging. The solvents were removed from the alcohol and ether extracts. The residues were combined, again dissolved in ether, and the insoluble part was removed by centrifuging.

Such ether extracts served for analysis. The ether extract obtained from protein P_1 was called E_2 and that from protein P_2 was E_4. The filtrates which remained after removal of proteins P_1 and P_2 were evaporated dry and the residue taken up in ether. The ether extract from the filtrate of P_1 was E_3 and from the filtrate of P_2, E_5.

Chemical Composition of the Bacterial Surface Layer

From 1240 mg of protein P_1 we obtained 124.1 mg of ether-soluble substance which was 10%. From 800 mg of protein P_2 we obtained 267 mg of ether soluble substance, that is, 33.37%. Proteins P_1 and P_2 gave a positive biuret reaction. The Bial reaction was positive only with protein P_1. In both P_1 and P_2 we determined quantitatively nitrogen, phosphorus, calcium, and magnesium (table). Also, by the spectrophotometric method we found 1% DNA in protein P_1.

By paper chromatography after hydrolysis we found in P_1 ribose and mannose, the latter in quantity many times surpassing that of ribose.

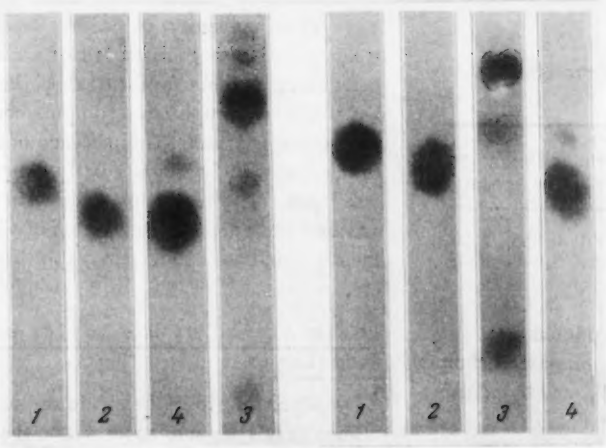

Fig. 1 Fig. 2

Fig. 1. Chromatogram of carbohydrates liberated after alkaline saponification of the lipids of preparation E_2. Designations: 1) glucose, 2) mannose, 3) carbohydrates liberated from lipid E_2. From above down: uronic acid, glucoseamine, trehalose, galactose, arabinose; 4) arabinose.

Fig. 2. Chromatogram of carbohydrates liberated after alkaline saponification of the lipids of preparation E_4. Designations: 1) glucose, 2) mannose, 3) carbohydrates liberated from lipid E_4. From above down: trehalose, galactose, glycerol ; 4) arabinose.

In both proteins after hydrolysis we found the amino acids lysine, aspartic acid, serine, glycine, glutamic acid, alanine, tyrosine, valine, phenylalanine, isoleucine, and leucine.

The ether soluble substances were studied by paper chromatography. In sample E_1 before and after saponification we found palmitic acid. In sample E_2 before hydrolysis we found oleic and myristic acids. After saponification we found a C_{20} acid, stearic, oleic, palmitic, and myristic acids, trehalose, and a small amount of hexuronic acid, hexoseamine, arabinose, and galactose (Fig. 1). In sample E_4 before and after saponification we found oleic, palmitic, stearic, and myristic acids. Carbohydrates in the form of trehalose and small amounts of glucose and galactose (Fig. 2) were observed only after saponification . In the composition of sample E_3 we found palmitic and myristic acids. In the composition of sample E_5 before saponification we found unsaturated C_{16} and C_{18} acids, saturated palmitic acid and myristic acids, and also trehalose and small amounts of mannose.

The resulting data permit us to consider that in the extraction of the bacteria by sodium taurocholate,the nucleic acids and proteins of the surface layer are removed. When the proteins are precipitated from solution by methyl alcohol, the nuclei acids precipitate along with the proteins (preparation P_1). When the proteins are precipitated by acetic acid, the nuclei acids are almost not precipitated (preparation P_2). The ether soluble substance is a collection of fatty acids and esters of these fatty acids and trehalose. The detection of mannose by paper chromatography after hydrolysis of the protein in much greater amount than the ribose evidently is related to the presence of a polysaccharide mannan, like that isolated earlier from diphtheria bacteria by Orlova [7]. The monosaccharides observed in small amount in the composition of sample E_4 after saponification evidently are products of splitting of a polysaccharide such as we [8] observed in the composition of the bound lipids. The passage of palmitic acid through the collodion membrane can be explained by the formation of a soluble complex with sodium taurocholate.

Since the esters of trehalose and the higher fatty acids are isolated from the bacteria along with the proteins, they precipitate with them and are not fully separated in the precipitation of the proteins from the solution by methyl alcohol and acetic acid, and perhaps they are bound with them in one complex.

TABLE

Chemical Composition of Proteins of the Bacterial Surface Layer

Protein	Amount in percent				
	N	P	Mg	Ca	water
P_1	12,8	0,4	0,051	0,055	1,96
P_2	12,39	0,05	0,010	0,046	0,28

Note: The protein was extracted with sodium taurocholate and precipitated from the extract by alcohol — P_1, and by acidification with acetic acid — P_2.

Thus, evidently, the surface layer of diphtheria bacteria is not a simple magnesium salt of a ribonucleoprotein, as had been thought before for the Gram-positive bacteria (Dubos et al.), but is a more complicated complex consisting of proteins, lipids, and polysaccharides. The presence of lipids in the surface layer of the cells itself permits a fairly easy removal of the so-called free lipids. The separation of these from the protein of this layer can explain the denaturing action of various factors on the proteins, factors which are due to removal of lipids (defatting, breaking up, etc.).

Toxic Action of the Lipids of the Bacterial Surface Layer

Since the lipids present in the surface layer of the diphtheria microbe cells are substances with a specific chemical composition, different from the lipids of higher organisms, we wished to study their action on the animal organism. For this purpose, rabbits and guinea pigs were injected subcutaneously with a vaseline oil solution of those fractions of the lipids of the surface layer of the bacteria which contained the trehalose esters of the higher fatty acids (E_2, E_4, E_5).

When 3-3.5 mg of these substances was injected we found swelling, hemorrhagic necrosis around the point of injection, and, finally, formation of a scab in this zone. The same experiment was carried out with lipids obtained by careful extraction of the bacteria with ligroin. (Seven-day cultures of the bacteria without destruction of the cells and without shaking were extracted by standing with ligroin. The ligroin was evaporated and the residue was used for analysis). The results were analogous to the results of the previous experiments.

In chemical composition these lipids were essentially the same esters, of higher fatty acids with trehalose.

Hence we can assume that the toxic action is due either to the free fatty acids or the esters of trehalose and the higher fatty acids. An explanation of this question is given by the study of the biological action of fraction G of the free lipids of diphtheria microbes which consists of the pure esters of the high molecular weight fatty acids and trehalose (the isolation and purification of this fraction was described earlier [9]). This fraction shows the same

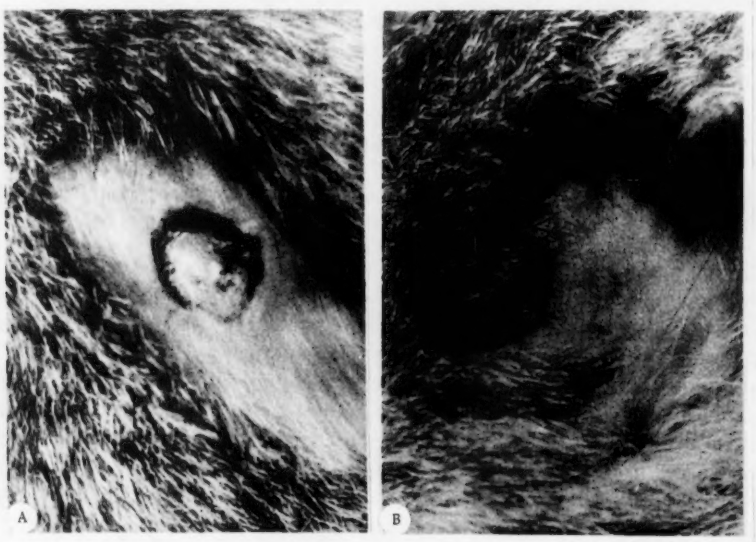

Fig. 3. Reactions after subcutaneous innection of the free lipids of fraction G. A) Injection of lipids of fraction G in paraffin oil; B) injection of paraffin oil (control).

action as the lipids obtained from the ligroin extract and the lipids of the surface layer of the bacterial cells.

Thus, for example, the subcutaneous injection of rabbits with 3.6 mg of this fraction in vaseline oil caused a circular hemorrhagic zone around the point of injection at a distance of 8 mm from the place of injection; the zone had a width of about 2 mm. Also, near the center was a sharp hemorrhagic spot. After several days this underwent necrosis and a scab formed on it (Fig. 3). At the point of injection of 0.5 ml of pure paraffin oil no change was observed.

Thus, the esters of trehalose and higher fatty acids, characteristic of the free lipids of diphtheria bacteria, after subcutaneous injection have a clear toxic action.

SUMMARY

By the action of a 2% solution of sodium taurocholate on the cells of diphtheria bacteria we have succeeded in separating the surface Gram-positive layer from the Gram-negative "skeleton".

We have isolated from the substances of the surface layer two proteins. From one of them (precipitated by methyl alcohol) which is a nucleoprotein, we have obtained 10% lipids by extraction. From the other (precipitated by acetic acid), which is almost free of nuclei acids, we have obtained 30% lipids by the same extraction. By paper chromatography we have established that these lipids are a mixture of free fatty acids and microsides (compounds of these acids with trehalose).

These microsides have a toxic action on the skin of animals after subcutaneous injection, causing swelling, necrosis, and finally formation of a scab in this zone.

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DETERMINATION OF AMINO SUGARS IN HYDROLYZATES OF ANIMAL, PLANT, AND BACTERIAL MATERIAL

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During the course of an investigation of certain plant and animal materials [1, 2] we found that a number of products of reaction of nitrogenous substances with carbohydrates may interfere with the determination of amino sugars by the method of Elson and Morgan [3], by giving similar color reactions. Such products arise chiefly in hydrolyzates or in aqueous extracts of the materials during heating with alkalis, both in the presence and in the absence of acetylacetone. We shall refer to these substances as type I condensates.

The results of our researches on the formation of type I condensates confirm the findings of other authors for a variety of materials, such as Neurospora crassa [4], the gelatinous capsule of echinoderm eggs [5], pineapple leaves [6], and model systems [7].

Such artifacts may also arise from condensates of nitrogenous substances with carbohydrates formed under the conditions of Elson and Morgan's procedure (heating in alkaline solution). The formation of such condensates (which we shall term type II condensates) proceeds very intensively during the drying of the material, and during extraction with hot alcohol, i.e., under dehydrating conditions at raised temperatures.

Type II condensates have a number of properties resembling those of hexosamines. Similarly to them, they undergo sorption on cationites, from which they may be readily eluted by solutions of HCl or NaCl, they are not precipitated by phosphotungstic acid, strong trichloroacetic acid (TCA) solutions, or saturated NaCl solutions, and they develop a coloration with p-dimethylaminobenzaldehyde only after reacting with acetylacetone in hot alkaline solution; they are highly resistant to acid hydrolysis under drastic conditions (6 N HCl, 6 hr at 100°).

Formation of types I and II condensates does not take place during acid hydrolysis (1 N HCl, 4 hr, and 6 N HCl, 6 hr at 100°).

Our modification of Elson and Morgan's procedure has been tested on a number of model systems and natural products, and has been found to eliminate interference with the determination of amino sugars in acid (6N and N HCl) hydrolyzates of biological materials.

EXPERIMENTAL PART

Our modification is based on the elimination from the hydrolyzates of substances which may be potential components of type I condensates. This is achieved by their precipitation from neutral or acid saturated NaCl solution by addition of strong TCA solution.

In some cases, we found that interfering nitrogenous substances present in the products of hydrolysis with N HCl were not fully eliminated by this treatment; treatment with phosphotungstic acid, which does not precipitate amino sugars, but which removes basic amino acids and peptides, was then necessary.

Elimination of the possibility of formation of type II condensates was achieved by the observance of controlled conditions of fixation and extraction of the material under analysis.

Fixation. The procedures adopted for fixation and extraction of the materials were based on the finding that type II condensates are not formed in acetone solutions in the presence of formaldehyde (probably because of blocking of the amino groups of the nitrogenous substances).

The following fixation procedures may be applied: 1) fixation of the comminuted material in acetone, cooled or at room temperature, with two changes of acetone (this process requires 1-2 days); 2) fixation in a 1: 5 formalin – 96% alcohol mixture, with cooling, at room temperature, or at the boiling point; 3) fixation of the comminuted material in TCA solution of a final concentration of 4-5% (taking into account the water content of the material), with cooling or at room temperature, with 2-3 rapid changes of the extracting solution, followed up by dehydration with acetone; 4) fixation with hot or cold 4-7% aqueous formaldehyde, changing the solution 2-3 times. Material fixed according to procedures 1 and 3 is dried in a desiccator or by exposure to the air. Formaldehyde must be eliminated from material fixed according to procedures 2 and 4, as it would interfere with subsequent determination of amino sugars.

Materials of a high lipid content (certain seeds, brain tissue, marrow, egg yolk, etc.) should be subjected to preliminary extraction with ether, chloroform, benzene, or petroleum ether.

Extraction. For the determination of the amino sugar content of high-molecular compounds the fixed and dried material is extracted 2-3 times with 20-30 volumes of boiling 1: 5 formalin – 96% alcohol mixture, or with cold 5% TCA solution. The extracted material is washed twice with acetone and once with ether, and is then dried.

Combined amino sugars may be determined in aqueous solutions or in TCA extracts. The extracts are evaporated down in a current of cold air from a fan, and the residues are analyzed for amino sugars after elimination of formaldehyde (by dialysis, or by washing the dry residue with acetone or ether).

Formation of type II condensates takes place after prolonged standing of aqueous extracts, irrespective of their pH.

For the determination of amino sugars in whole blood, serum, plasma, synovial fluid, urine, and other body fluids, leaf and root sap, and bacterial and mold culture media, we take 4-5 ml of the supernate from centrifugation, and add to it an equal volume of 2 N or concentrated HCl; the resulting solution is hydrolyzed.

Basic Reagent (BR) – Aqueous NaOH – NaCl – CCl_3COOH Solution

The composition of the BR solution is such that, when mixed with a corresponding volume of 6N HCl, formation of acid or alkaline concentrated or saturated NaCl solutions (final NaCl solutions) takes place. TCA separates from such solutions in the form of a heavy oily layer, together with which are removed substances interfering with amino sugar determination, including colored hydrolysis products. The supernatant, clear, almost colorless phase is free of interfering substances, and is taken for determination of amino sugars.

Should the final NaCl solution be too alkaline, separation of the bottom phase, and with it of interfering substances, does not take place. Addition to the systems of small portions of NaOH or Na_2CO_3 leads to the gradual, and finally complete, dissolution of the bottom phase, and with it of the interfering hydrolysis products. A similar may be observed in systems containing 80-90% TCA in saturated NaCl solution.

The efficacy of the BR, for eliminating interfering substances, rises parallel with the final NaCl concentration, to a maximum at saturation. On the other hand, the degree of purification of the hydrolyzates rises parallel with the TCA content of a given volume of BR.

Hexosamines are distributed unevenly between the top and bottom phases, being concentrated in the former. The glucosamine content of both phases, and the volume of each phase, sould hence be measured, in experiments in which the proportions of the BR components are fixed, for a series of solutions of pure glucosamine in 6 N HCl. The glucosamine contents may be determined in aliquots of the top and bottom layers; it is, however, preferable to arrive at these values indirectly, by a method based on the constancy of the partition coefficient of glucosamine between the two phases, for a BR of a given composition.

The glucosamine content of test solutions can thus be derived from the results of its determination in the upper layers of the test and of standard solutions (solutions of glucosamine in 6 N HCl), treated with BR.

From the difference between the hexosamine content of the whole of the standard solution and that of its upper layer alone, the hexosamine content of the bottom layer is given by :

$$c_2 = \frac{cv - c_1 v_1}{v_2}, \qquad (I)$$

where v_1 and v_2 are the volumes of the top and bottom layers, respectively, c_1 and c_2 are the glucosamine concentrations of these phases, v is the initial volume of standard solution, and c is its hexosamine concentration.

The partition coefficient K of hexosamine between the phases may hence readily be derived, as $K = c_1/c_2$ and the hexosamine content of the bottom layer is expressed as $c_2 = c_1/K$.

The total hexosamine content, X, of the sample of hydrolyzate under analysis is then given by:

$$X = c_1 v_1 + \frac{c_1 v_2}{K} \qquad (II)$$

The BR should be tested before use. This is done by shaking the appropriate volumes of BR and 6 N HCl. Should the bottom phase not form under these conditions (owing to the excessive alkalinity of the final NaCl solution), concentrated HCl is added, in the least amount needed to give the maximum volume of bottom phase. The corresponding volume of concentrated HCl is then added to the bulk of the reagent.

Preparation of the BR. 32 g of NaOH is dissolved in 100 ml of half-saturated NaCl. The solution is cooled to room temperature, and is then added in small portions, together with the sediment forming, to 100 ml of 85% TCA, cooling the beaker in a stream of tap water. The mixture is filtered through glass-wool, after cooling to room temperature. The reagent is stored at room temperature in dark glass bottles.

Determination of Amino Sugars in 6 N HCl Hydrolyzates

Hydrolysis with 6 N HCl liberates amino sugars from both readily and difficultly hydrolyzable hexosamine-containing substances. For the determination of amino sugars, a weighed amount (10-60 mg) of the powdered, dry residue remaining after fixation and extraction of the material is hydrolyzed for 6 hr with 6 N HCl, in a test tube fitted with a reflux condenser.

A mixture of 2 ml of the decanted or filtered hydrolyzate* with 3 ml of BR is shaken. The volume of the bottom phase forming is 1.9 ml, and of the top layer 3.3 ml. The concentration of glucosamine in the bottom layer was 40% of that in the upper layer, with the given BR.

The mixture is allowed to stand for 15-20 min, after which 0.5-1 ml portions of the upper layer are transferred to tubes graduated at 8 and 10 ml, and neutralized with $NaHCO_3$. If the upper layer is turbid it should be filtered through ordinary filter paper.

The amino sugar content of the hydrolyzate is then determined, by the method of Elson and Morgan, measuring the optical density at 530 mμ (green filter) 20-30 min after addition of the p-dimethylaminobenzaldehyde reagent. One of the test solutions (acetylacetone not added) is taken as the blank.

Analyses involving addition of known amounts of pure glucosamine to the biological material were performed in order to assess to what extent breakdown of hexosamines took place during hydrolysis. These experiments were performed as follows. Solutions containing 2-4 mg of glucosamine hydrochloride were added to weighed samples (60 mg per 10 ml of 6 N HCl) of a series of products of animal, plant, and bacterial origin, and of model systems (casein – starch mixtures, in the proportions 1:4 and 1:1). The products were hydrolyzed, and hexosamines were determined in the hydrolyzates by the above method. The results of the analyses showed that not less than 70% of the added glucosamine was accounted for.

Experiments in which solutions of glucosamine hydrochloride (0.2-0.4 mg per ml) were treated with 6 N HCl (6 hr at 100°) showed that a certain proportion of the glucosamine (18%) underwent breakdown, due to the action of the strong acid used. There is therefore an unavoidable, relatively small loss of hexosamines, due to their destruction during the hydrolytic process by the strong acid used, and to their condensation with decomposition products of the biological material during its hydrolysis.

Determination of Amino Sugars in N HCl Hydrolyzates

The free amino sugars present in the products of hydrolysis in N HCl originate from readily hydrolyzable hexosamine-containing compounds.

* The BR may be added direct to the hydrolysis tube, when the volume of hydrolyzate is small.

Two variants of our method may be applied to the determination of these amino sugars:

Variant A. 1. A weighed sample of the material, or an aliquot of its extracts, is hydrolyzed on a boiling water bath for 4 hr with 6 ml of N HCl.

2. One ml of decanted or filtered hydrolyzate is mixed with 1 ml of concentrated HCl, 3ml of BR is added, and the mixture is shaken. The subsequent analytical procedure is as for hydrolyzates in 6 N HCl.

We apply this variant to most biological materials, in particular to those of animal origin taken for clinical investigations; its applicability is shown by the results of our analyses of 30 specimens of material of animal, plant, and bacterial origin, and of model mixtures (casein – starch, casein – sucrose). Variant A cannot be applied if coloration appears in the blank systems containing 0.5 N Na_2CO_3 instead of acetylacetone.

Variant B. 1. The sample is hydrolyzed for 4 hr on a boiling water bath with 10.5 ml of N HCl.

2. To 7 ml of the decanted or filtered hydrolyzate is added 0.5ml of a saturated solution of phosphotungstic acid, the mixture is stirred, stood for 30–60 min, and then filtered on a Buchner funnel through ordinary filter paper.

Should the filtrate be turbid, filtration through the same paper is repeated one or more times.

3. To 1 ml of filtrate are added 1 ml of concentrated HCl and 3 ml of BR. The subsequent operations are as for hydrolysis with 6 N HCl.

Variant B is of universal applicability for the determination of amino sugars in the products of hydrolysis of any kind of biological material in N HCl, although it need not be used for many kinds of material. We found that it had to be applied to the analysis of bean seeds and of corn pollen, which are distingiushed by their high contents of readily hydrolyzable heteropolysaccharides and of proteins.

About 80% of glucosamine (2–4 mg) added to the samples could be accounted for, using variants A or B.

SUMMARY

Analytical procedures have been developed, serving for the determination of amino sugars in hydrolyzates of animal, plant, and bacterial material in 6 N or N HCl; the procedures require the preliminary fixation and extraction of the material, and the elimination of interfering substances from the hydrolyzates. Fixation and extraction of the material involve the use of acetone and of aqueous or alcoholic solutions of formalin; their object is to prevent the formation of condensates of nitrogenous products with carbohydrates, which might give similar color reactions to the Elson – Morgan reaction for amino sugars. Purification of the hydrolyzates depends on removal of substances interfering with determination of amino sugars (carbohydrates, nitrogenous compounds), by addition of concentrated TCA solutions to the neutral or acid solution saturated with respect to NaCl. This is achieved by adding to the product of hydrolysis with 6 N HCl an appropriate volume of TCA – NaOH – NaCl solution; when hydrolysis is effected with N HCl, the hydrolyzate is first mixed with an equal volume of concentrated HCl. With certain types of plant material additional purification of the products of hydrolysis with N HCl, involving addition of phosphotungstic acid, is recommended.

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COMPARATIVE STUDY OF THE TYPES OF BONDS FORMING DURING IN VITRO AND IN VIVO INCORPORATION OF AMINO ACIDS INTO PROTEINS

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A large number of papers dealing with the process whereby amino acids are incorporated into isolated proteins have been published [3-10], since the elucidation of this process [1, 2]. Special attention was devoted in these researches to the nature of the bonds forming when free amino acids undergo incorporation into isolated proteins.

Comparative data derived from the partial alkaline or acid hydrolysis of myosin labeled in vivo or in vitro with C^{14}-glycine or -tyrosine showed that the bonds arising during incorporation of free amino acids into isolated proteins or into body proteins are of uniform strength [11]. Partial acid or alkaline hydrolysis of proteins cannot, however, enable the nature of the bonds formed in vitro or in vivo between the free amino acids and the other components of proteins to be compared. It is, for this purpose, necessary to use proteolytic enzymes as the hydrolyzing agents acting on specific peptide bonds.

In the present paper we describe the results of a study of the nature of the bonds formed during incorporation of free C^{14}-tyrosine and C^{14}-glycine from the medium into isolated myosin and albumin, based on the partial enzymic hydrolysis of the resulting proteins. We found that under these conditions the amino acids are joined to the proteins by α-peptide linkages, in the same way as when these amino acids are incorporated into the proteins in the intact organism.

MATERIALS AND METHODS

In our experiments we used solutions of native myosin and serum albumin, labeled in vivo and in vitro. Myosin labeled in vivo was prepared according to the procedure described in reference [11], with the difference that it was not treated with TCA. Myosin labeled in vitro was also prepared according to the directions of reference [11], except that after incubation it was not precipitated and washed with TCA, but was freed of radioactive contaminants by repeated dissolution in KCl solution, followed by precipitation in distilled water, until free radioactivity was no longer found in the supernate.

For the preparation of serum albumin labeled in vivo we administered C^{14}-tyrosine and C^{14}-glycine to rabbits [11]. We isolated labeled serum albumin from the serum of these animals by the method of Kosner and Debro [12]. For the preparation of in vitro labeled albumin we added 1-2 μC of C^{14}-tyrosine to 30-40 ml of blood serum; somewhat higher dosage levels were taken for labeling with C^{14}-glycine. The serum was incubated with the labeled amino acids for 2-3 hr at 37°, after which albumin was isolated from it by the above-cited method [12], with the difference that, after the first precipitation of serum proteins by adding an equal volume of 10% TCA, and before dissolving the protein in alcohol, we washed the precipitate with 5-8 portions of 5% TCA containing 0.1% of the unlabeled amino acid, for removal of free radioactivity. In our experiments we used aqueous solutions of freshly prepared albumin or of lyophilized albumin. The purity and native condition of the preparations were checked by means of solubility and electrophoretic mobility measurements [12].

TABLE 1

Mean Values of Radioactivity of Myosin and Albumin Labeled In Vivo and In Vitro at Different Stages of the Process of Partial Enzymic Hydrolysis

Time of digestion hr	Extent of hydrolysis, %, from		C^{14}-labeled myosin						C^{14}-labeled glycine					
			specific activity		total activity of protein residue imp/min		fall in total activity, %		specific activity		total activity of protein residue imp/min		fall in total activity, %	
	nonprotein nitrogen	tyrosine	in vivo	in vitro	in vivo	in vitro	in vivo	in vitro	in vivo	in vitro	in vivo	in vitro	in vivo	in vitro
0	0	0	98	93	1007	396	—	—	97	75	1636	264	—	—
3	49	43	139	242	734	242	29	39	131	92	1690	210	0	20
6	72	67	213	—	628	—	39	—	141	—	953	—	42	—
24	93	92	234	273	223	55	78	86	160	210	533	114	68	57
	Albumin labeled with C^{14}-tyrosine								Albumin labeled with C^{14}-glycine					
0	0	0	364	200	1128	1139	—	—	347	274	1672	1287	—	—
24	25	30	365	160	790	676	36	31	250	194	882	684	48	47
48	52	50	367	150	477	450	58	61	250	160	562	360	77	72
72	69	60	321	156	417	327	63	72	273	160	409	240	76	81
96	82	80	313	195	313	272	74	76	260	170	221	144	92	89

* Total radioactivity of residual protein calculated on the protein content per ml of digest.

The stability of the bonds formed during incorporation of the amino acids into the proteins was checked by reprecipitating the proteins from alkaline solutions, and treating them with ninhydrin [7]. We found no significant lowering of the radioactivity of myosin or albumin, labeled in vivo or in vitro, as a result of these operations.

Proteins labeled in vivo or in vitro with C^{14}-tyrosine or C^{14}-glycine were subjected to the action of trypsin in buffer of pH 8.6. For the digestion we usually took 50 ml of KCl solution containing 8-12 mg of myosin per ml. In the experiments on albumin we took 40 ml of 0.6-08% aqueous solution of protein. To these solutions we added 25 ml of 0.2 M borate buffer of pH 8.6 , and 10mg of highly purified trypsin. After 0.3, 6, and 24 hr of incubation of the myosin systems, and after 0.24 , 48, 72 and 96 hr of incubation of the albumin systems, 10-15 ml samples of proteolyzate were taken, and proteins were precipitated with TCA . The protein precipitates were washed and dried , and their radioactivity was measured according to the directions of reference [7], using and end-window counter. The extent of hydrolysis of the proteins at the different stages of digestion was assessed from measurements of the nonprotein nitrogen content of the filtrates, by Conway's modification of the Kjeldahl method, and of nonprotein tyrosine, using the Folin reagent.

In addition, we measured the radioactivity of the free amino acid and peptide fractions of the TCA filtrates of the samples taken after the above-specified digestion times. The radioactivity of the free amino acid fraction of the enzymic hydrolyzates was measured in the precipitare of barium carbonate obtained after treating 1 ml of hydrolyzate with ninhydrin [13]. The radioactivity of the peptide fraction of the hydrolyzates was derived as the difference between the total activity of 1 ml of hydrolyzate and that of its amino acid fraction.

RESULTS

The results of experiments on the enzymic hydrolysis of myosin labeled in vivo and in vitro with C^{14}-tyrosine and C^{14}-glycine are shown in Table 1. Radioactivity is retained by the protein residues of myosin throughout the whole of the proteolytic process, irrespective of whether the labeled amino acids had been incorporated in vivo or in vitro.

TABLE 2

Radioactivity of the Free Amino Acid and Peptide Fractions of Tryptic Hydrolyzates of Proteins Labeled In Vivo an In Vitro

Time of digestion, hr	Protein decomposition %, calculated on N content	Myosin, labeled in vivo: C^{14}-tyrosine: Radioactivity of the hydrolyzate: total, imp/min·ml	C^{14}-tyrosine: free amino acid fraction, %	C^{14}-tyrosine: peptide fraction, %	C^{14}-glycine: total, imp/min·ml	C^{14}-glycine: free amino acid fraction, %	C^{14}-glycine: peptide fraction, %
0	0	0	0	0	0	0	0
3	60	93	0	100	141	0	100
6	67	244	10,6	89,4	1181	0	100
24	95	495	19,1	80,9	1180	0	100
0	0	—	—	—	0	0	0
3	0	—	—	—	0	0	0
6	32	—	—	—	128	0	100
24	73	—	—	—	1025	0	100
30	91	—	—	—	1697	0	100
Myosin, labeled in vitro							
0	0	28	0	100	0	0	0
3	72	74	13,5	86,5	30	0	100
6	—	—	—	—	—	—	—
24	98	261	10,3	89,7	235	0	100
0	0	—	—	—	0	0	0
3	44	—	—	—	145	0	100
6	—	—	—	—	—	—	—
24	82	—	—	—	190	0	100

Time of digestion, hr	Protein decomposition %, calc. on N content	Albumin, labeled in vivo: C^{14}-tyrosine: Radioactivity of the hydrolyzate: total, imp/min·ml	C^{14}-tyrosine: free amino acid fraction, %	C^{14}-tyrosine: peptide fraction, %	C^{14}-glycine: total, imp/min·ml	C^{14}-glycine: free amino acid fraction, %	C^{14}-glycine: peptide fraction, %
0	0	0	0	0	0	0	0
24	35	398	9,8	90,2	1400	2,0	98,0
48	—	—	—	—	—	—	—
72	64	810	7,7	92,3	2196	3	97,0
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
Albumin, labeled in vitro							
0	0	60	0	100	0	0	0
24	30	693	5,26	94,74	—	—	—
48	40	667	11,8	88,2	482	3,8	96,2
72	60	854	12,8	87,2	804	3,4	96,6
96	70	882	11,0	89,0	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—

As proteolysis proceeds, the specific activity of myosin rises in all the experiments, and after 24 hours, when the extent of hydrolysis, as assessed from the nonprotein tyrosine values, amounts to 90-95%, it is more than 2-3 times as great as initially. This is evidence of the uneven distribution of the incorporated C^{14}-tyrosine and C^{14}-glycine within the myosin molecule. It is possible that incorporation of these amino acids is smaller at those parts of the myosin molecule which are most readily attacked by trypsin. The uneven distribution of the labeled amino acids may be associated with unequal rates of renewal of tyrosine units in different myosin fractions.

Notwithstanding the rise in specific activity of the residual protein during the course of the proteolytic reaction, the total radioactivity of these residues falls steadily, although at a considerably lower rate than the rise in nonprotein nitrogen content of the hydrolyzates.

As is evident from Table 1, the radioactivity of albumin, similarly to myosin, is retained in the residual protein at all stages of proteolysis, irrespective of whether the albumin had been labeled in vivo or in vitro. In contrast to the results for myosin, however, the specific activity of the residual albumin fractions labeled with C^{14}-glycine falls in most cases during the initial stages of digestion. Accordingly, the total radioactivity of the protein residue falls faster than the nonprotein content of the hydrolyzate rises. These findings show that the C^{14}-glycine incorporated in vivo or in vitro into serum albumin is not distributed uniformly throughout the molecule, but takes place most intensively at those parts of the albumin molecule which are the most readily attacked by trypsin. Experiments with albumin into which C^{14}-tyrosine had been incorporated did not give evidence of uneven distribution of the labeled amino acid.

The changes in radioactivity taking place during the enzymic hydrolysis of myosin and albumin are characteristic of each of these proteins; whether the labeled amino acids were incorporated in vivo or in vitro did not, however, significantly affect the results obtained.

We measured the radioactivity of the free amino acid and peptide fractions of the tryptic hydrolyzates of myosin and albumin, with the object of making a more detailed comparison of the nature of the chemical bonds forming during the process of incorporation of free amino acids into proteins in vivo and in vitro. When myosin labeled with C^{14}-tyrosine in vivo is subjected to the action of trypsin (Table 2), 10-19% of the total radioactivity of the hydrolyzate is present in the free amino acid fraction, which is evidence of splitting off from the myosin molecule of the tyrosine which had been added to it during its incorporation into the proteins of the intact organism with formation of α-peptide bonds. The same distribution of radioactivity was found in hydrolyzates of myosin labeled with C^{14}-tyrosine in vitro. In this series of experiments the radioactivity of the free amino acid fraction amounted to 10-13% of the total radioactivity of the tryptic hydrolyzate.

The free amino acid fraction of the hydrolyzate of myosin labeled in vivo or in vitro with C^{14}-glycine did not possess any radioactivity. All the radioactivity of the hydrolyzate was found in the peptide fraction. C^{14}-glycine is not split off from the protein by the action of trypsin. The same result was obtained irrespective of whether incorporation of the labeled amino acid had taken place in the intact organism, or in an isolated amino acid — protein system.

Free C^{14}-tyrosine was split off from albumin labeled in vivo or in vitro with this amino acid, during digestion with trypsin. In both series of experiments the radioactivity of the free amino acid fraction amounted to about 10% of that of the tryptic hydrolyzate. In experiments with albumin labeled with C^{14}-glycine in vivo or in vitro, the content of labeled amino acid fraction of the hydrolyzate was only about a third of the value found for protein labeled with C^{14}-tyrosine. Thus trypsin liberates C^{14}-tyrosine or C^{14}-glycine from albumin labeled with these amino acids, irrespective of whether incorporation had taken place in the intact animal or in an isolated system. This effect was, however, smaller for glycine-labeled albumin.

Our findings show clearly that the bonds formed during incorporation of C^{14}-tyrosine or C^{14}-glycine into myosin or albumin, whether in the intact animal or in isolated protein — amino acid systems, are equally susceptible to attack by trypsin. It may hence be concluded that the bonds formed when C^{14}-tyrosine is incorporated into isolated myosin ot albumin are analogous to those formed when these amino acids are incorporated into myosin or albumin in the intact organism. It may, moreover, be concluded that the sites of incorporation of C^{14}-tyrosine and C^{14}-glycine into myosin or albumin in vitro resemble those of incorporation in vivo.

Our experimental findings indicate that the distribution within the protein molecule of amino acids incorporated into it in vivo or in vitro is determined by the chemical structure and properties of the protein concerned.

SUMMARY

Comparative studies have been made of the nature of the bonds formed when free amino acids are incorporated into myosin and albumin, in intact animals and in isolated protein — amino acid systems.

C^{14}-tyrosine and C^{14}-glycine were found to be incorporated at the same sites of the protein molecule, both in vivo and in vitro. Incorpoation of C^{14} tyrosine into myosin, and of C^{14} tyrosine and C^{14}-glycine into serum albumin, involves formation of α-peptide linkages, both in isolated systems and in vivo.

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EFFECTS OF SOME DRUGS ON THE RESPIRATION, ADENOSINE TRIPHOSPHATE CONTENT, AND PHOSPHOPROTEIN METABOLISM OF SLICES OF RAT CEREBRAL CORTEX

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The metabolism of phosphoproteins (PhP) has been found to be closely correlated with nerve cell activities. Incorporation of P^{32} from inorganic phosphate present in the incubation medium, into phosphoproteins of rat cerebral cortex slices was found to take place only when oxidation of glucose, coupled with phosphorylation of ADP, was proceeding vigorously in the cells [1, 2]. Under certain experimental conditions, however, it was found possible to suppress PhP metabolism (as assessed from the rate of incorporation of(P^{32} into PhP), although oxidative phosphorylation was still proceeding at a high rate. Measurements of the rate of turnover of phosphoprotein phosphorus made it possible to perceive disturbances in the vital activities of nerve cells, even when these cells seemed to be functioning normally in other respects (oxygen uptake and reconstitution of ATP).

The rapid and sensitive response of phosphoproteins to changes in the conditions of incubation of brain slices could be used as an indicator of the functional state of nerve cells. In this connection it was of interest to ascertain whether this indicator could be applied to the investigation of the mode of action of certain drugs which acted selectively on the nervous system.

The drugs used for the treatment of diseases of the nervous system are usually classified in three groups: 1) inhibitors (depressants); 2) tranquilizers (sedatives); 3) stimulators (analeptics). This classification is based on the nature of the effect of these drugs, and each group includes substances of the most diverse chemical structure The literature dealing with the mode of action on the nervous system of drugs belonging to these three groups is fairly extensive [3, 4]. There are, however, very few papers dealing with the effects of these agents on any particular biochemical process. Hence only for a few of the drugs is the mechanism of their action understood.

In the present research we examined the effect of representatives of each class of drug on the incorporation ofP^{32} into phosphoproteins of rat brain slices. Of the depressive drugs we examined medinal and phenylurethane; of the analeptics, phenamine, corazone, and strychnine; of the tranquilizers, reserpine and aminazine.

METHODS AND MATERIALS

Selection of concentration of drug. For most of the drugs examined by us the dosage levels which would affect intravellular processes in vitro were not known. The dosage levels adopted in our experiments were based on the therapeutic dosage.

We calculated the concentrations of drugs used in our in vitro experiments on the basis that immediately after its administration the therapeutic dose is diluted in 8 liters of fluid (5 liters of blood and 3 liters of lymph; for this rough calculation we ignored any subsequent unevenness in distribution of the drug in the organs and tissues of the body). This was usually regarded as being the minimum dosage level; in addition we examined the effects of doses exceeding it by 1-3 orders of magnitude.

Measurement of respiration of the slices. Rat brain slices were cut with a safety blade [2]. The slices were rinsed in cooled McIlwain solution [5] before adding them to the systems. Into the main compartment of

the Warburg flasks we introduced 6 mg of glucose (33 μ moles), the solution of the drug, about 0.1 g of brain slices, and McIlwain solution to a final volume of 2.5 ml. Incubation was conducted for 60 min at 37°. The results are expressed as μ l of oxygen absorbed per 100 mg of fresh weight of tissue per hour.

The effect of the drugs on cell respiration was first examined in all cases, as we could find no information in the literarure as to the permeability of cell membranes to most of them. If the presence of a given drug had any effect on respiration, we took this to be evidence that it had entered the cells. Apart from this, measurement of respiration served for the more precise selection of concentrations of drugs, since those based on the above calculation gave only a very rough idea of what the effective dose should be. As the maximum dose, we took the least concentration of drug which depressed respiration; apart from this, we examined the effects of 2-3 concentrations not affecting respiration. Earlier papers had shown [1,6] that there was a connection between PhP metabolism and oxidative phosphorylation. Inhibition of the latter was invariably associated with inhibition of incorporation of P^{32} into PhP. For this reason, as a second preliminary test we determined the ATP content we could draw conclusions as to whether the action of the given drug was exerted on the oxidative phosphorylation system, or in some other way.

Determination of relative specific radioactivity (RSR) of phosphoproteins.

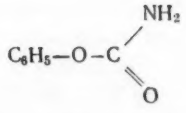
The RSR of PhP is expressed by the ratio $\frac{\text{specific radioactivity of PhP}}{\text{specific radioactivity of } P_i}$, where specific radioactivity is imp/ min·μ g of phosphorus of the given fraction.

The amount and the radioactivity of intracellular inorganic phosphorus (P_i) were measured by the methods described in an earlier paper [1]. PhP phosphorus was determined by a method based on that of Schmidt and Thannhauser, with certain modifications [2], , and with extraction of the phosphomolybdate complex with isobutyl alcohol, as recommended by Vladimirov et al. [7]. The optical density of the isobutyl alcohol solution was measured with a photoelectric colorimeter, using an S656 light filter. For the measurement of the radioactivity of the phosphorus of the PhP we took 2 ml portions of each solution, after reading its optical density, placed it on a round filter paper on a glass slide, allowed it to dry, and counted the number of impulses per minute with a counter placed above the paper.

RESULTS

Depressants

Phenylurethane



(Phenyl carbamate)

Urethane (ethyl carbamate), which is closely related to phenylurethane, has been used medicinally as a hypnotic and sedative. Both urethane and phenylurethane are employed as laboratory narcotics. Their effect in vitro on the mechanisms of biochemical cell processes has not been investigated until now.

Our experiments on phenylurethane gave unambiguous results: at a concentration of 5×10^{-4} M it had practically no effect on the uptake of oxygen by the slices, but it caused some inhibition of ATP resynthesis and of phosphoprotein metabolism (Table 1). At a concentration of 2.5×10^{-3}, respiration, ATP resynthesis, and PhP metabolism were all affected. Finally, all of these activities were conduced to a minimum at the highest concentrations examined (5×10^{-3} and 5×10^{-2} M).

The wide range of values found for ATP content in the absence of the drug is noteworthy. In the given series of experiments, the ATP contents of slices incubated without phenylurethane ranged from 1.3 to 6.7 μg of 10-minute phosphorus per 100 mg of fresh tissue; this constitutes a fivefold difference between extreme values, whereas the highest values found for resporation were only about 50% above the lowest. This is evidence of the lability of the oxidative phosphorylation system, as compared with that regulating the uptake of oxygen by the cells.

TABLE 1

Effect of Various Concentrations of Phenylurethane on the Respiration ATP Content and Phosphoprotein Metabolism of Slices of Rat Cerebral Cortex

Respiration: μ l O_2/100 mg tissue·hr; ATP: μg 10-min P/100 mg tisue·hr; PhP: RSR

No. of expt.	Test	No phenylurethane	Concentration of phenylurethane		
			$5{\cdot}10^{-4}$ M	$2{,}5{\cdot}10^{-3}$ M	$5{\cdot}10^{-3}$ M
1	Respiration	121	123	78	42
2	ATP	3,9	2,0	—	0,8
3	ATP	1,3	1,6	0,5	0,3
	RSR of PhP	0,11	0,05	0,06	0,02
4	ATP	2,5	1,7	1,3	0,6
	RSR of PhP	0,09	0,11	0,06	0,01
5	ATP	1,9	1,5	1,4	0,3
	RSR of PhP	0,09	0,04	0,03	0,02
6	ATP	2,6	2,3	2,1	1,4
7	ATP	6,7	6,0	4,4	1,6
	RSR of PhP	0,28	0,21	—	0,04

TABLE 2

Effect of Various Concentrations of Medinal on the Respiration ATP Content and Phosphoprotein Metabolism of Slices of Rat Cerebral Cortex

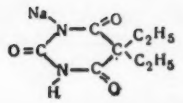
The units are the same as in Table 1

No. of expt.	Test	No medinal	Concentration of medinal			
			10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M
1	Respiration	100	97	107	76	65
2	ATP	3,4	3,9	3,7	—	3,0
	RSR of PhP	0,10	0,09	0,13	—	0,05
3	ATP	3,2	—	3,2	4,1	3,2
	RSR of PhP	0,12	—	0,15	0,15	0,12
4	ATP	4,3	—	4,1	—	—
	RSR of PhP	0,10	—	0,19	—	—
5	ATP	3,1	—	3,5	—	—
	RSR of PhP	0,09	—	0,13	—	—
6	ATP	3,7	—	3,5	—	—
	RSR of PhP	0,09	—	0,12	—	—
7	ATP	2,7	—	1,6	—	—
	RSR of PhP	0,09	—	0,08	—	—
8	ATP	2,0	—	2,3	—	—
	RSR of PhP	0,07	—	0,06	—	—
9	ATP	2,8	—	2,7	—	—
	RSR of PhP	0,09	—	0,09	—	—
10	ATP	3,1	—	2,8	—	—
	RSR of PhP	0,09	—	0,09	—	—

Differences in the physiological state of the animals, variations in the length of time elapsing between killing the animals and starting incubation, and minor differences in the speed of preparation of the slices and in the way in which they were prepared, had little effect on respiration, but considerably affected the ability of the cells to resynthesize high-energy substances. The fall in ATP content of cells at lower concentrations of phenylurethane (5×10^{-4} M) than affected respiration (Table 1, experiments 1-6) can also be ascribed to the higher lability of the oxidative phosphorylation system.

As we found in our earlier researches [1, 2, 6] there is a definite connection between ATP resynthesis and PhP metabolism (the latter is appreciable only when oxidative phosphorylation is proceeding intensively). Concentrations of phenylurethane which inhibited oxidative phosphorylation also inhibited incorporation of P^{32} into PhP.

Medinal



(Sodium 5,5-diethylbarbiturate)

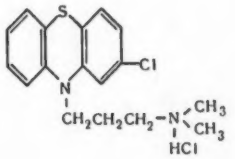
Medinal is a hypnotic and tranquilizing drug. At the ordinary dosage levels it retards the respiratory rate, and lowers blood pressure. High doses of the drug depress respiration [9]. Administration of narcotic doses to rats is followed by a rise in their brain phosphocreatine content [10, 11]. Toxic doses of barbiturate (nembutal) lower the ATP and phosphocreatine contents of the brain of rats [10].

The depressive effect of medinal on respiration became evident in our in vitro experiments at a concentration of 10^{-3} M. No inhibitory effect could be perceived at a concentration of 10^{-4} M. Since the greatest interest was attached to the highest concentrations of narcotic which did not affect respiration, most of our experiments with medinal were performed at this concentration of 10^{-4} M. The results are presented in Table 2.

In general, the ATP content of the slices was unaffected by the addition of medinal to the medium. The RSR of phosphoproteins was either unaffected by the presence of 10^{-4} M medinal, or rose very slightly. The oxidative phosphorylation system is more sensitive than is the respiratory system to the action of phenylurethane. However, this narcotic depresses PhP metabolism to the same extent as it does ATP resynthesis. This was not the case with medinal, although, together with phenylurethane, it belongs to the class of depressants. ATP and PhP metabolism were not affected by 10^{-2} M medinal, at which concentration we found definite depression of respiration. Whereas phenylurethane inhibits incorporation of P^{32} into phosphoproteins of rat brain slices indirectly, by interfering with oxidative phosphorylation, the mode of action of medinal must be different, and cannot involve the oxidative phosphorylation system.

Tranquilizers

Aminazine (chlorpromazine)



[2-Chloro-N-(3-dimethylaminopropyl) phenothiazine hydrochloride]

Aminazine is one of the basic representatives of the new group of drugs, which have been termed neuroplegic (nerve-blocking) agents. This class of drugs has a sedative action, and its members exerts a variety of complex effects on the functions of the central and peripheral nervous systems. A general sedative effect follows the administration of aminazine, and a state resembling physiological sleep is induced by appropriate doses of the drug [9]. Addition of aminazine in low concentrations to suspensions of brain mitochondria [12] or to brain homogenates [13] or slices [14] uncouples phosphorylation from respiration. Incorporation of glycine into the proteins of brain slices was inhibited at the same concentrations [14]. Respiration is also inhibited at higher concentrations of the drug. This effect may be due to inhibition of enzymes of the flavoprotein group [15]. The work of Decsi et al. [16-18] has shown that the central ganglion blocking effect of aminazine is related to simultaneous inhibition of phosphorylation in the respiratory chain and of ATP-ase; according to Decsi, inhibition of phosphorylation is due to blocking of tissue sulhydryl groups by aminazine.

We examined the effect of aminazine (at concentrations of 4×10^{-6} to 5×10^{-3} M) on the respiration, ATP content, and PhP metabolism of brain slices. At a concentration of 2.5×10^{-3} M, aminazine powerfully depresses respiration, completely inhibits accumulation of ATP, and reduces the RSR of PhP to a minimum (Table 3).

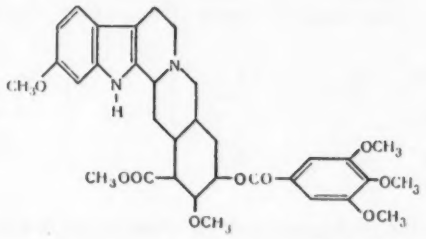
TABLE 3

Effect of Various Concentrations of Aminazine on the Respiration ATP Content and Phosphoprotein Metabolism of Slices of Rat Cerebral Cortex
The units are the same as in Table 1

No. of expt.	Test	No aminazine	Concentration of aminazine			
			1–2·10⁻⁴ M	5·10⁻⁴ M	10⁻³ M	2,5–5·10⁻³ M
1	Respiration	118	96	104	49	23
2	ATP	5,7	4,8	4,0	—	0,9
	RSR of PhP	0,05	0,05	0,04	—	0,03
3	ATP	7,3	7,3	6,2	—	0,1
	RSR of PhP	0,18	0,13	0,10	—	0,06
4	ATP	5, 7	7,2	—	2,3	0,9
	RSR of PhP	0,08	0,08	—	0,04	0,01
5	ATP	6,2	4,2	—	1,9	0,5
	RSR of PhP	0,10	0,10	—	0,01	0,00

At a concentration of 5×10^{-4}, the drug had no appreciable effect on respiration, but it slightly depressed ATP resynthesis. A tendency toward depression of PhP metabolism was also evident (Table 3). It may be concluded that aminazine is able to penetrate the cell membrane, and to affect intracellular processes. Depression of PhP metabolism in the presence of aminazine may be related to uncoupling of oxidative phosphorylation.

Reserpine



Reserpine is also a member of the class of neuroplegic and sedative drugs, and it is now widely applied in medicine. In our experiments, reserpine had practically no effect on the respiration, the oxidative phosphorylation, or the phosphoprotein metabolism of rat brain slices, at any of the concentrations tested (from 4×10^{-5} to 4×10^{-7} M*); see Table 4.

TABLE 4

Effect of Reserpine on the Respiration ATP Resynthesis and Phosphoprotein Metabolism of Slices of Rat Cerebral Cortex
The units are the same as in Table 1

No. of expt.	Test	No reserpine	Concentration of reserpine		
			4·10⁻⁷ M	4·10⁻⁶ M	4·10⁻⁵ M
1	Respiration	92	122	91	79
2	ATP	1,8	2,7	2,6	2,7
	RSR of PhP	0,09	—	0,07	0,08
3	ATP	2,8	2,5	3,0	2,9
	RSR of PhP	0,07	0,07	0,08	0,06

* In view of the low solubility of reserpine in water, we dissolved a tablet containing 25 mg of reserpine in 10 ml of ethanol. One ml of the alcoholic solution was diluted with McIlwain's solution, to give a concentration of 4×10^{-5} M.

This result may be ascribed to the inability of reserpine to penetrate the cell membranes in vitro, and hence to affect the enzyme systems examined by us. McIlwain [19] believes that the pharmacological action of reserpine may be ascribed to its releasing certain amines from body depots, and that these amines act specifically on the brain.

Analeptics

Strychnine

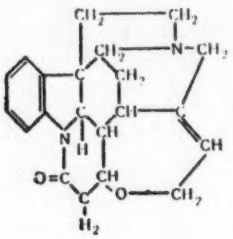


TABLE 5

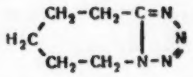
Effect of Various Concentrations of Strychnine on the Respiration ATP Content and Phosphoprotein Metabolism of Slices of Rat Cerebral Cortex
The units are the same as in Table 1

No. of expt.	Test	No strychnine	Concentration of strychnine			
			$7 \cdot 10^{-4} M$	$7 \cdot 10^{-4} M$	$7 \cdot 10^{-4} M$	$7 \cdot 10^{-5} M$
1	Respiration	88	91	—	100	93
2	ATP	3,1	—	—	3,3	3,6
	RSR of PhP	—	—	0,06	0,05	0,08
3	ATP	3,2	3,2	—	4,2	3,1
	RSR of PhP	0,10	0,14	—	0,13	0,12

Our experiments showed that the respiration of slices of brain cortex was not depressed by strychnine, even at the highest concentrations tested (3.5×10^{-3} M), which were practically saturated solutions of the alkaloid in McIlwain's solution (Table 5).

Nor was any effect on the ATP content or the PhP metabolism of the slices observed. The impression received is that strychnine is not able to penetrate the cell membranes, under our experimental conditions. It would otherwise be difficult to explain how it is that so powerful a stimulating agent as is strychnine could have no effect on the cell metabolic processes examined by us.

Corazole



(Pentamethylenetetrazole)

Corazole has a stimulating action on the central nervous system, and it also stimulates respiration and circulation; at high dosage levels it acts as a convulsant [9]. Palladin et al. [20, 21] have shown that during stimulation by corazole the ATP content of rabbit brain at first rises slightly, and then falls considerably. Administration to rabbits of pervitin (acting very similarly to phenamine) causes a gradual, steady rise in the ATP content of the brain. As these authors point out, the biochemical changes correspond to the physiological effects of the drugs: pervitin (and phenamine) stimulate the central nervous system, remove fatigue, and raise work capacity, whereas corazole, which stimulates the cerebral cortex, does not raise the working capacity of the brain.

TABLE 6

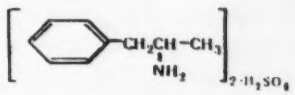
Effect of Various Concentrations of Corazole on the Respiration and Phosphoprotein Metabolism of Slices of Rat Cerebral Cortex
The units are the same as in Table 1

No. of expt.	Test	No corazole	Concentration of corazole		
			10^{-4} M	10^{-3} M	10^{-2} M
1	Respiration	168	—	156	170
2	RSR of PhP	0,04	0,04	0,06	0,05

At the concentrations taken, corazole did not affect the respiration of the slices, and had no regular effect on PhP metabolism (Table 6).

It must hence be concluded either that corazole does not penetrate the cell membrane, or that it does not, in fact, exert its action under the conditions of our experiment.

Phenamine



D,L-β-phenylisopropylamine sulfate

Phenamine is a very active stimulator of the central nervous system. At the appropriate dosage levels, it raises the excitability of the respiratory center, enhances the rate and the amplitude of respiration, intensifies excitatory processes, and quickly and effectively abolishes the feeling of tiredness. It is believed [9] that the stimulatory effect of phenamine on the central nervous system may be ascribed to its inhibitory effect on amine oxidase, thus preventing accumulation of aldehydes in nerve tissues; these aldehydes are believed to have a depressive effect on oxidative processes.

TABLE 7

Effect of Various Concentrations of Phenamine on the Respiration ATP Content and Phosphoprotein Metabolism of Slices of Rat Cerebral Cortex
The units are the same as in Table 1

No. of expt.	Test	No phenamine	Concentration of phenamine		
			10^{-4} M	10^{-3} M	10^{-2} M
1	Respiration	150	140	156	88
2	ATP	2,0	1,8	1,8	1,5
	RSR of PhP	0,06	0,07	—	—
3	ATP	2,0	2,6	2,5	1,3
	RSR of PhP	0,05	0,06		0,07
4	ATP	2,1	2,1	3,3	1,3
	RSR of PhP	0,06	0,10	0,06	0,04
5	ATP	1,8	2,1	2,3	1,5
	RSR of PhP	0,11	0,11	0,11	0,09
6	ATP	5,3	4,7	5,2	2,7
	RSR of PhP	0,17	0,16	0,18	0,13
7	ATP	4,0	—	6,2	2,5
	RSR of PhP	0,06	—	0,14	0,07
8	ATP	4,7	—	4,1	—
	RSR of PhP	0,14	—	0,16	—
9	ATP	4,5	—	3,9	—
	RSR of PhP	0,15	—	0,16	—

Our experiments revealed differences between the action of phenamine on metabolic processes in brain slices, according to whether high or low dosage levels were taken. At a concentration of 10^{-2}M phenamine slightly depressed respiration, ATP resynthesis (Table 7, experiments 2, 3, 4, 5 and 6), and PhP metabolism (experiments 4, 5 and 6).

At concentrations of 10^{-2}M phenamine causes increase in cell membrane permeability, as shown by a rise in the specific radioactivity of intracellular inorganic phosphate (Table 8). Practically no change was found in the O_2 uptake of the slices in the presence of 10^{-4} to 10^{-3} M phenamine and the same applied to cell membrane permeability, whereas there was a certain activation of ATP resynthesis and of phosphoprotein metabolism.

TABLE 8

Specific Radioactivity of Intracellular Inorganic Phosphate in the Presence of Phenamine imp/min·μg P

Without phenamine	10^{-4} M phenamine
127	154
108	136
212	266
215	227

DISCUSSION OF RESULTS

The wide use in medicine of drugs acting specifically on the nervous system, for narcosis or for therapeutic purposes, justifies the interest attached to the elucidation of their mechanism of action on the organism. Substances belonging to a wide variety of chemical classes, and prossessing the most diverse structures, have been found to exhibit pharmacological activity with respect to the central nervous system. The pharmacological activity of these substances is related to their ability to react with one or another of the biochemical systems of the nerve cells. A significant contribution to the elucidation of the mechanisms of such reactions is made by in vitro experiments, since in vivo experiments only permit the observation of the final stages of the action of the drugs on metabolism; this actios is frequently complex and indirect.

It is of interest to compare the therapeutic dosage levels of the drugs examined by us with those found effective in our model experiments with brain slices. The relevant data are presented in Table 9.

The high sensitivity of the intact organism to the action of a number of drugs may be ascribed to a number of causes. The effect of a drug may be determined by its action on some central mechanism. Thus it is known that phenamine acts specifically on the respiratory center. It is obvious that no such action could be expected in tissued isolated from the organism as a whole. On the other hand, some drugs act selectively on certain metabolic steps. These may not have been elements of the metabolic pathways studied by us. It should,finally, be borne in mind that cell membrane permeability is an active process, depending on the physiological condition of the tissues. Cell membrane permeability in the intact organism may differ substantially from that observed in tissue slices. It is conceivable that the absence of any evidence of pharmacological activity of reserpine, strychnine, and corazole in our experiments was due to their inability to penetrate the cell membrane.

The aim of this research was to establish the effects of different classes of drugs on PhP metabolism. Our other analyses were, as has been mentioned, of a subsidiary nature. Our reasons for undertaking this investigation were, firstly, our earlier findings [1, 2, 6], establishing the lability of this group of proteins (or, at least, of their phosphorus-containing moiety), and, secondly, the increasing tendency toward explaining the activity of drugs (in particular, of narcotics) in terms of their effects on protein components of cells. According to Karasik [22]: "We can now not only state that proteins.....play a basic role in the reactions of the organism with narcotic drugs, but we are also able to describe the changes which may take place in the proteins as a result of these reactions".

Our experiments did not reveal any specific effects of the drugs examined on the metabolic rate of the phosphoprotein fraction. Three of the drugs (reserpine, strychnine, corazole) showed no effect, and one (phenamine) depressed PhP metabolism simultaneously with respiration of the slices and oxidative phosphorylation. In low concentrations (10^{-4} to 10^{-3}M), phenamine had no effect on respiration, but showed a tendency toward stimulation of ATP resynthesis and of PhP metabolism. High concentrations of phenylurethane (2.5×10^{-3}M or higher) inhibited all three processes. A characteristic feature of the action of phenylurethane was that it affected respiration less than it did the other two processes. Depression of respiration was not found in 5×10^{-4}M phenylurethane, whereas inhibition of ATP resynthesis and of PhP metabolism were clearly evident. Medinal and aminazine resembled each other in that in their presence PhP metabolism did not vary strictly parallel with changes in respiration. However, whereas a concentration of medinal which depressed respiration (10^{-3}M) did

TABLE 9

Dosage of Drugs

Drug	Concen. in body fluids after administ. of therapeutic dose	Expt. with slices of cerebral cortex — Concentration inhibiting		
		respiration	accumulation of ATP	PhP metabolism
Medinal	$6 \cdot 10^{-4}$ M	10^{-3} M	No action	
Phenylurethane		$2{,}5 \cdot 10^{-3}$ M	$5 \cdot 10^{-4}$ M	$5 \cdot 10^{-4}$ M
Phenamine	$4{,}2 \cdot 10^{-6}$ M	10^{-2} M	10^{-2} M	10^{-2} M
Corazole	$8 \cdot 10^{-5}$ M	No action		
Strychnine	$7 \cdot 10^{-7}$ M	No action		
Reserpine	$2 \cdot 10^{-7}$ M	No action		
Aminazine	$1{,}8 \cdot 10^{-5}$ M	10^{-3} M	10^{-3} M	$5 \cdot 10^{-4}$—10^{-3} M

* The therapeutic doses were taken from Mashkovskii's book [9]. The calculations were based on the premise that the dose administered was distributed uniformly in 5 liters of blood and 3 liters of lymph.

TABLE 10

Effects of Some Drugs on Intracellular Metabolic Processes in Slices of Rat Cerebral Cortex

Concentration	Respiration		ATP resynthesis		PhP metabolism	
	depression	stimulation	depression	stimulation	depression	stimulation
Phenamine						
10^{-4} M	—	—	—	+?	—	+?
10^{-3} M	—	—	—	+?	—	+?
10^{-2} M	++	—	++	—	+?	—
Phenylurethane						
$5 \cdot 10^{-4}$ M	—	—	+	—	+	—
$2{,}5 \cdot 10^{-3}$ M	+	—	++	—	++	—
$5 \cdot 10^{-3}$ M	++	—	+++	—	+++	—
Aminazine						
1—$2 \cdot 10^{-4}$ M	—	—	+?	—	+	—
$5 \cdot 10^{-4}$ M	—	—	+	—	+	—
10^{-3} M	+	—	++	—	+++	—
$2{,}5 \cdot 10^{-3}$ M	++	—	+++	—	+++	—
Medinal						
10^{-5} M	—	—	—	+?	—	—
10^{-4} M	—	—	—	+?	—	+?
10^{-3} M	+	—	—	—	—	+?
10^{-2} M	++	—	—	—	+?	+?

Designation of symbols: —, no effect; +?, effect not regularly observed in all the experiments; +, definite, but weak, action; ++, quite definite action; +++, powerful action.

not depress the ATP content or the metabolic rate of PhP (at this, and at the lower concentration of 10^{-4}M, medinal tended to stimulate PhP metabolism), aminazine, on the other hand, depressed PhP metabolism and ATP resynthesis at concentrations which did not affect respiration.

Thus in all those cases in which a drug was found to affect PhP metabolism, it was also found to affect resynthesis of ATP, i.e., it affected the mechanism of oxidative phosphorylation. This correlation was less close

for medinal, whence it might be supposed that this drug has a direct action on PhP metabolism, not implicating oxidative phosphorylation.

Our findings for those drugs showed any effects on intracellular processes taking place in brain slices are summarized in Table 10.

At the concentrations taken, phenylurethane and aminazine gave only depressive effects. Low concentrations of medinal and phenamine tended to stimulate resynthesis of ATP and PhP metabolism. It hence follows that the inclusion of a drug into the classes of stimulating or depressive agents by no means implies that, in the former case, it should be expected to have a depressive effect on intracellular processes proceeding in vitro, or that a stimulatory effect should be found in the latter case. Medinal belongs to the class of depressor drugs, yet under appropriate conditions it can stimulate metabolic processes. The stimulatory drug phenamine can, similarly, depress metabolic activity when present in the appropriate concentrations.

SUMMARY

A study has been made of the effects on metabolic processes proceeding in rat brain slices of three classes of drugs acting on the central nervous system — depressors, stimulators, and sedatives.

Three of the seven drugs examined (corazole, strychnine, and reserpine), did not, at the concentrations taken (10^{-4} to 10^{-2} M, 7×10^{-6} to 7×10^{-3} M, and 4×10^{-7} to 4×10^{-5} M, respectively), have any effect on the respiration, ATP resynthesis, or phosphoprotein metabolism of rat brain slices. This finding may be ascribed either to the inability of these drugs to penetrate the cell membrane, or to the absence of any effect of the drugs on the processes studied. At a concentration of 10^{-3} M medinal depressed the respiration of the slices; 10^{-4} M medinal had no effect on respiration, but tended to stimulate ATP resynthesis and PhP metabolism.

Depression of PhP metabolism by phenylurethane was closely paralleled by depression of oxidative phosphorylation. At a concentration which depressed respiration (2.5×10^{-3} M), phenylurethane also depressed the other two processes examined.

The central nervous system stimulator phenamine depressed respiration, ATP resynthesis, and PhP metabolism, at a concentration of 10^{-2} M. At lower concentrations (10^{-4} to 10^{-3} M) it had no effect on the rate of uptake of axygen by the brain slices, but tenden to stimulate ATP resynthesis and PhP metabolism.

Aminazine, which has a sedative effect in the intact animal depressed respiration, ATP resynthesis, and PhP metabolism of brain slices, at a concentration of 10^{-3} M.

No direct connection could be discerned between the effects on these metabolic processes of the drugs examined and their chemical structure or the nature of their pharmacological action.

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ELECTROPHORETIC STUDY OF THE PROTEIN FRACTION OF THE BLOOD SERUM OF CARP PRODUCERS AND THEIR OFFSPRING

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Although the problem of the inheritance of acquired immunity in fish is of great practical importance and scientific value, it has not been studied until recently.

There is much work in the literature on the question of inherited acquired immunity in mammals and birds [1-5].

When he considered this question from the evolutionary viewpoint, Zil'ber [6] concluded from the literature data that antibodies are of comparatively recent phylogenetic origin as immunological protective factors which appear in the vertebrates and reach their development in warm-blooded animals. According to the data of most authors [7-11], in cold-blooded vertebrates (frogs, turtles, fish), antibodies are formed poorly, slowly, and in low quantity, and so under natural conditions can hardly play the part of immunological protective factors. However, Friedberger and Seeling [12] and also Mutomo [13] found an antibody effect in the blood of cold-blooded vertebrates. They found normal hemolysins in toads, and also in eels and many other varieties of fish. Lazar [14] in a study of agglutinins in frog blood serum showed a hemolysin of complex structure for cold-blooded animals and considered it to be identical with the hemolysin of warm-blooded animals. Shvartsman [15] found that frogs can form antibodies when immunized. Svet-Moldavskii [16] showed that reptiles have the ability to produce virus-neutralizing antibodies when the body temperature is raised. Ranke [17] compared the electrophoretograms of the proteins of carp blood serum for carp with rubeola and for healthy carp and showed the decrease of some protein fractions in the blood of fish attacked by rubeola.

In seeking for an effective prophylaxis and medicinal agent against carp rubeola, the most dangerous disease of pond fish, we carried out work to study the effect of vaccination of the producers on the resistance of the offspring to rubeola. In this paper we give the results of an electrophoretic investigation of the proteins of blood serum from carp producers which has been immunized and their offspring, carried out by us in 1957-1958.

METHODS

The work was carried out on carp grown in the Angelin hatchery, Krasnodar region. A portion of the carp producers were vaccinated twice in the autum and twice in the following spring, several days before spawning. At the same time, as controls in spawning, we took a portion of unvaccinated producers attacked by an acute form of rubeola.

After spawning, the young were transferred to a growing pond and after hibernation, in the spring the yearlings were placed in a developing pond where, in the course of the second year they attained commercial weight.

The experiments with vaccinated carp producers were carried out in 1955-1956 and 1957-1958. Each year we vaccinated new producers and the carp vaccinated before were injected with unweakened vaccine (virus).

TABLE 1

Fractional Composition of Blood Serum in Carp Producers under Different Conditions

No.	Characteristics of subject	Total protein	γ-Globulin		β-Globulin		α-Globulin		Albumin	
		%	%	g	%	g	%	g	%	g
1	Healthy, before beginning hibernation	3,84	9,6	0,37	24,6	0,94	43,8	1,71	22,0	0,86
2	Healthy, from vaccinated producers before hibernation	5,25	26,0	1,37	26,7	1,40	23,3	1,22	24,0	1,26
3	Healthy, after hibernation	2,73	24,5	0,66	34,2	0,93	26,3	0,71	15,0	0,42
4	Healthy, from vaccinated producers, after hibernation	2,92	34,6	1,0	28,6	0,88	27,0	0,79	9,8	0,28
5	Brought to the limits of exhaustion	2,0	57,4	1,15	18,3	0,37	16,6	0,33	7,7	0,15
6	Very acute form of rubeola	1,35	15,0	0,19	35,4	0,44	43,5	0,55	6,1	0,07

We called the latter fish hyperimmunized. The vaccinated and hyperimmunized carp producers were healthy for three years, stood the vaccination well, and spawned very well.

The offspring from the immunized producers also developed well and as a rule showed growth above the standard. When there was a severe outbreak of rubeola, the yearlings from the vaccinated and hyperimmunized producers bore the disease well and showed little loss, while the young from the unvaccinated producers all died. During the first winter and the second year of life of the fish from the vaccinated and hyperimmunized producers there was almost no occurrence of rubeola and the fish showed good development and growth.

For the preparation of the vaccine, blood was taken with a sterile Pasteur pipet from the heart of carp of different ages with evidences of attack by acute rubeola. At the same time we took transudate from various parts of the skin (air bladder). The transudate and the blood in equal amounts were placed in small sterile glass flasks in which previously we had placed calcined sterile sand to defibrinate the blood. The mixture was energetically shaken and after defibrination was poured in equal parts into two sterile flasks. For the preparation of the weakened vaccine, we added to one flask a sterile physiological saline solution (0.64% NaCl) and 2% formalin in the amounts 100 ml of mixture + 100 ml of physiological saline + 4 ml of formalin solution. In the second flask we added to the mixture only sterile physiological saline (1:1) without the formalin. This mixture, which we called unweakened virus, was used for hyperimmunization. The sterility of the solutions was tested by seeding on meat-peptone agar and meat-peptone broth.

Blood was taken from the fish heart with a sterile Pasteur pipet for the electrophoretic investigation. The total protein concentration in the serum was determined refractometrically. The fish serum proteins were fractionated by paper electrophoresis [18]. Coloring of the strips and washing out of the color were carried out by the method described by Gencks, Getton, and Durrum [19]. Quantitative study of the electrophoretogram was carried out by the method of elution and following colorimetric determination of the amount of protein in the eluate [18,20].

RESULTS

In healthy one-year-old carp and in mature individuals we clearly showed four main fractions of the serum proteins: albumins and α-, β-, and γ-globulins. As we showed earlier [21], the chief fractions normally consist of two subfractions of albumin, four subfractions of α-globulin (α_1, α_2, α_3, and α_4), and two subfractions of β-globulin (β_1 and β_2); the γ-globulin fraction is electrophoretically homogeneous.

During hibernation under natural conditions, when the carp are starved for 6-7 months, along with decrease is muscle fat, carbohydrate, and protein, we also find a decrease in total protein concentration in the serum by 1-1.5 % (absolute). This decrease occurs, first, because of albumin, and to a lesser extent because of α- and β-

TABLE 2

Fractional Composition of Blood Serum Proteins in Vaccinated and Unvaccinated Carp Producers

Characteristics of subject	Total protein	γ-Globulin		β-Globulin		α-Globulin		Albumin	
	%	%	g	%	g	%	g	%	g
Unvaccinated	2,68	18,8	0,51	33,4	0,89	22,6	0,61	25,2	0,67
Vaccinated (twice)	6,16	55,8	3,44	31,6	1,94	6,6	0,41	6,0	0,37
Hyperimmunized	6,88	55,1	3,8	14,3	0,99	26,2	1,77	4,4	0,32

globulin; the content of the γ-globulin fraction increases both relatively and absolutely. Thus, for example, with long starvation (Table 1, 5) the total protein concentration in the blood decreases twofold compared to the initial value. The amount of β-globulin decreases threefold, of α-globulin and albumin, more than fivefold, while the content of γ-globulin triples.

In one-year-old carp with acute rubeola, the total serum protein content decreases. The decrease is preferentially due to albumin, in less degree to α- and β-globulin, and also, in contradiction to what occurs in starvation of healthy carp, because of decrease in the γ-globulin fraction (Table 1, 6). This fact leads us to believe that rubeola suppresses the mechanism of formation of γ-globulin in fish.

In the vaccinated and hyperimmunized carp producers the quantity of protein in the blood serum is considerably greater than in the unvaccinated fish (Table 2). Characteristic changes occur in the fractional composition: in the vaccinated producers there is an increase in γ-globulin along with a sharp decrease in the albumin and α-globulin fractions. In the hyperimmunized carp, besides the sharp increase in γ-globulin, there is a comparatively high content of α-globulin; the amount of albumin (4.4%) is also decreased in them.

The offspring of the first generation, 1-3 years of age, from the vaccinated and hyperimmunized producers, easily withstood rubeola and showed goog body growth. The protein content of the blood serum was high as a rule (Tables 3 and 4). After hibernation, at the end of April, these individuals showed a decreased serum protein, mostly due to albumin. However, the content of the γ-globulin fraction in their serum protein remained high. During the summer the total protein concentration in the one-year-old carp serum doubled (Table 3). The ratio of the protein fractions changed: the albumin content increased (24-30%), α-globulin (23-26%) and β-globulin (26.6%); the concentration of γ-globulin fell 1-1.5 times (17.3-26.0%). At the same time, both the relative and absolute content of γ-globulin remained considerably higher (4.8%) than in healthy one-year-old carp from unvaccinated producers (Table 3).

In Table 4 we give data on two year old carp which earlier had easily withstood rubeola and whose weight during the developing period reached 700 g; the total protein content was 6.12%. It is interesting to see that the amount of the γ-globulin protein fraction in certain individuals is more than twice as great as the γ-globulin content in other fish, and reaches 73.9% (4.52 g), while the protein concentration in other fractions is comparatively low: albumin is 3.6% (0.22 g), α-globulin 9.7% (0.6 g), β-globulin 12.9% (0.8 g).

We obtained similar results in our experiments in other fish in the middle and at the end of the development period. We can assume that introduction of rubeola-causing organisms into carp from vaccinated producers greatly stimulates the reticuloendothelial system with the formation of antibodies (γ-globulins).

DISCUSSION

In the present study we have established by the method of electrophoretic analysis some characteristics of the content of protein fraction in the blood serum of immunized fish. These characteristics allow us to draw preliminary conclusions as to the nature and significance of the different protein fractions in the development of immunity in fish. Comparison of the electrophoretograms obtained in the study of blood serum of artificially immunized carp producers and control unvaccinated fish has shown that in the blood of the first group and in even greater degree in the blood of their offspring there is a large increase in proteins of the γ-globulin fraction.

TABLE 3

Fractional Composition of Blood Serum Proteins in Yearling Carp of the First Generation from Vaccinated Producers

Subjects and date of analysis	Total protein %	γ-Globulin		β-Globulin		α-Globulin		Albumin	
		%	g.	%	g	%	g	%	g
Unvaccinated, blood taken October 20, 1958	3,76	4,8	0,18	33,8	1,77	30,0	1,13	31,4	1,18
Vaccinated, blood taken July 26, 1958	4,9	17,3	0,85	26,5	1,30	26,2	1,28	30,0	1,47
Vaccinated at end of hibernation, blood taken April 24, 1958	2,92	34,6	1,0	28,6	0,88	27,0	0,79	9,8	0,28
Vaccinated, blood taken October 20, 1958	5,25	26,0	1,37	26,7	1,40	23,3	1,22	24,0	1,26

TABLE 4

Fractional Composition of Blood Serum Proteins in Two-Year-Old Carp of the First Generation from Vaccinated Producers

Subject and date of analysis	Total protein %	γ-Globulin		β-Globulin		α-Globulin		Albumin	
		%	g	%	g	%	g	%	g
Mirror carp. Blood taken July 21, 1958	5,25	18,6	0,98	32,1	1,69	26,5	1,38	22,8	1,20
Scaled carp. Blood taken July 21, 1958	5,22	18,1	0,94	27,9	1,46	24,4	1,29	29,5	1,53
Blood taken July 21, 1958	5,03	38,5	1,94	24,9	1,25	24,7	1,25	11,9	0,59
Blood taken July 21, 1958	5,51	25,9	1,43	24,4	1,35	25,6	1,40	24,1	1,33
Blood taken July 29, 1958	6,12	73,9	4,52	12,9	0,79	9,7	0,60	3,5	0,21

We earlier [22] showed that in healthy two-year-old carp, among which rubeola had not been observed, the relative amount of γ-globulin fraction in the serum protein was not high and varied in the range 9-14 %, which is three or four times less than in carp artificially immunized and 1.5-2 times less than in carp of the control group, which withstood rubeola.

At the present time there is enough fundamental evidence to consider antibodies as modified serum globulins [23-28]. If this is so, then on the basis of the data we have obtained we can say that in vaccinated and hyperimmunized carp producers (in greater degree in these latter), and in their offspring, there is generated a protective function against rubeola. Thus in fish, as in warm-blooded animals, antibodies are evidently formed in the blood serum proteins in immunization, and these are found in the γ-globulin fraction. The high content of the γ-globulin fraction in most of the vaccinated carp producers and their offspring is maintained for a long time. This is perhaps because the immunity acquired by vaccination is retained under the conditions of the Angelin fish hatchery by natural immunization (contact with fish attacked by rubeola).

The positive experiments with vaccinated carp producers against rubeola leads us to begin a wide experimental study of the use of vaccinated producers in fish against other infectious diseases.

SUMMARY

Vaccination of carp producers against rubeola protects them from the disease. The offspring obtained from immunized producers inherit this acquired immunity to rubeola and show resistance to the disease. The increase in amount of γ-globulins in the blood serum of carp producers after vaccination and of their rubeola resistant offspring evidently depends on the formation of antibodies.

From these data we can recommended the use of vaccination of carp producers also for other fisheries where rubeola is a fish disease.

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THE MOLECULAR WEIGHT AND ENZYMATIC ACTIVITY OF PROTEOLYTIC ENZYMES TREATED WITH ULTRASONIC WAVES

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A number of enzymes dissolved in water saturated with air are inactivated by ultrasonic waves. Under these conditions there is inactivation of desoxyribonuclease [1], invertase [2], hyaluronidase [3], pepsin [4], and other enzymes. In the opinion of most authors, inactivation of enzymes in the ultrasonic field depends on the fact that in ultrasonic treated water the various groups which bear the enzyme properties of the protein are oxidized. The occurrence of oxidative processes is connected with the appearance in the treated water of the radicals.

$$OH \text{ and } HO_2 ; H_2O \rightarrow H + OH ; H + O_2 \rightarrow HO_2$$

The process of splitting the water molecules occurs in a cavitation field under the influence of a rapidly moving electrical charge [5,6,7].

In the present paper we give data which show that in the ultrasonic field there is oxidation not only of the terminal or side groups of the proteins, but, under definite conditions, change in the dimensions of the protein molecule. The sound-treated protein solutions when oxygen is present in the water show splitting of the molecules into the smallest stable particles with the lowest molecular weight. In the presence of hydrogen, on the contrary, there is an increase in the protein molecule which is indicated by an increasing molecular weight of the protein.

We have obtained analogous data in the study of proteolytic enzymes treated with ultra sound in the presence of these gases. We have been able to establish definite regularities which indicate the role of some gases (active and inert) in the initiation or the inhibition of chemical processes which usually lead to inactivation of the enzyme under investigation.

EXPERIMENTAL PART AND DISCUSSION OF RESULTS

As the objects of investigation we used horse serum albumin twice recrystallized and lyophilically dried, pepsin obtained by the method of Northrop and Kunitz as modified by Levdikova [8], and trypsin, isolated according to Northrop and Kunitz. Water solutions of serum albumin and pepsin and a solution of trypsin in 0.0025 N HCl were treated ultrasonically.

Conditions of treatment: 740 kilocycles frequency of oscillation, and 10 watts/cm^2 intensity. Ultrasonic treatment of these solutions was carried out in a special glass apparatus at room temperature (20-25°), which was maintained by cooling the vessel undergoing ultrasonic treatment by running tap water. The treated solutions were first saturated with nitrogen, hydrogen, helium, and argon. These gases were carefully freed from traces of oxygen by passing them through a special column as constructed by Kantorovich and Rapoport [9]. As a rule, according to polatographic analysis, oxygen was absent from water saturated with gases purified in this way.

The molecular weight of the serum albumin, tripsin, and pepsin was determined with Wilhelmi surface weights by the method of Bull [10]. Definite concentrations of protein were introduced on the surface with a micro-pipet; the volume of added drop was determined by the weight method.

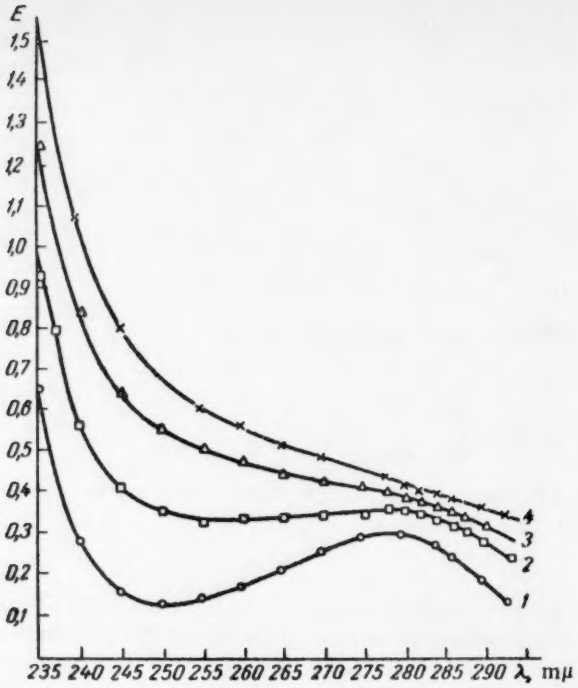


Fig. 1. Relation of optical density of pepsin to length of treatment in water saturated with air. Enzyme concentration 0.02%: 1) untreated; 2) treated 15 min; 3) treated 30 min; 4) treated 60 min.

TABLE 1

Relation of Molecular Weight of Serum Albumin to Length of Action of Ultrasonic Waves

Duration of ultrasonic treatment, min	Molecular weight of protein	Change in molecular weight, %
0	76 000	0
5	70 300	— 9
10	62 000	—24
12	61 700	—24
24	38 000	—50
50	37 800	—49

The vehicle for the serum albumin was a 5% solution of $(NH_4)_2SO_4$, pH 1.8; the required pH was reached by adding HCl. (Ammonium sulfate was first freed from surface-active contaminants with activated charcoal, and then recrystallized three times). The vehicle for the trypsin was also 5% $(NH_4)_2SO_4$, but the pH of this solution was brought to 8.7 by adding 5 N NaOH to it.

The concentration of protein after treatment by ultrasound was determined by the method of Lowry [11] using Folin phenol reagent. Actually we showed that change in concentration did not occur even after treatment for an hour (0.08% solution of serum albumin).

The enzymatic activity of trypsin was determined by the rate of hydrolysis of serum albumin, and that of pepsin by the rate of hydrolysis of hemoglobin.

RESULTS

As Table 1 shows, the molecular weight of serum albumin decreases within definite limits with increasing duration of ultrasonic treatment in air. After 50 min of treatment of molecular weight of the protein has decreased by almost 50%. It is apparant that such a marked decrease in the molecular weight cannot be explained by the splitting off of some side or terminal group. Also the molecular fragments which are formed still have a considerable molecular weight and do not lose the ability to form monolayers.

Another result was obtained when the solutions were first saturated with other gases: nitrogen, helium, and hydrogen (Table 2). In the presence of helium or nitrogen in the treated solution (25 min period of treatment), the initial molecular weight of the protein remained unchanged. Only after longer exposure (50 min) when the solution was saturated with nitrogen did we observe some decrease in molecular weight of serum albumin (by

TABLE 2

Change in Molecular Weight of Serum Albumin Treated with Ultrasonic Waves in the Presence of Various Gases

Duration of ultrasonic treatment, min	Gas which saturates protein solution	Molecular weight of protein before and after	Change in molecular weight, % of initial value
0	Air	76 000	0
25	"	38 000	—50
0	Helium	76 000	0
25	"	78 700	0
0	Hydrogen	73 400	0
25	"	122 600	65
0	Nitrogen	76 000	0
25	"	76 000	0
50	"	59 000	—24

about 24%). In the presence of hydrogen, as a rule, there was an increase in molecular weight of the ultrasonically treated protein. The solution remained perfectly clear and the treated protein did not lose its ability to spread on the surface of the liquids which we used. On the average, under the conditions mentioned, the molecular weight of the albumin increased about 65%.

The action of ultrasonic waves on proteolytic enzymes was analogous.

As Table 3 shows, depending on the nature of the gas, present in the treated solutions, there is a decrease or an increase in the molecular weight of the enzymes studied. It was also shown that the nature of the gas which saturated the solution determined the enzymatic activity of the protein studied. It was shown that the trypsin molecule is less stable to the action of ultrasonic waves than is the pepsin molecule. Thus, for example, in the presence of air, under the action of ultrasonic waves (duration of treatment 15 min), the enzymatic activity of trypsin falls by 75%, and of pepsin, by only 38%. However, under these conditions, the molecular weights of these enzymes decrease by the same amount, namely, by 13%.

There was a parallel decrease in the optical density in the ultraviolet spectra of the enzymes treated in the presence of air. This effect was strengthened with increased duration of treatment (Fig. 1).

It is noteworthy that a decrease in enzyme activity is found when the action of the ultrasonic waves causes a decrease also in the molecular weight of the treated protein. In turn, the decreased molecular weight occurs when there is a definite existence of oxidizing processes in the ultrasonic field, that is, under conditions in which oxygen or free hydroxyl radicals are present in the treated liquid as products of the splitting of the water molecule.

The role of the free hydroxyl radicals in the oxidation of proteins, which is accompanied by a decrease in molecular weight and enzyme activity, is shown by studies of protein solutions which are treated with ultrasonic waves in the presence of argon. As Table 3 shows, argon, as distinguished from helium, even when oxygen is absent from the treated solution, does not prevent the splitting of the proteins and inactivation of pepsin. It should be noted that in solutions of pepsin and trypsin treated in the presence of oxygen or argon there is a decrease in absorption maximum in the ultraviolet field which is specific for proteins. In the presence of hydrogen and helium

TABLE 3

Change in Molecular Weight and Activity of Pepsin and Trypsin, Treated in Water Saturated with Different Gases

Enzyme	Duration of treatment, min	Gas which saturateds enzyme solution	Molecular weight of enzyme before and after	Change in molecular weight, %	Change in enzyme activity, %
Pepsin	0	Air	37 700	0	0
"	15	"	32 700	—14	—38
"	0	Hydrogen	37 800	0	0
"	15	"	41 000	8	0
"	30	"	41 000	8	0
"	30	Argon	32 100	—13	—30
"	30	Helium	37 000	0	0
Trypsin	0	Air	37 700	0	0
"	15	"	32 700	—13	—75
"	30	"	28 000	—24	—85
"	0	Hydrogen	37 700	0	0
"	30	"	49 200	30	0
"	60	"	49 200	30	0

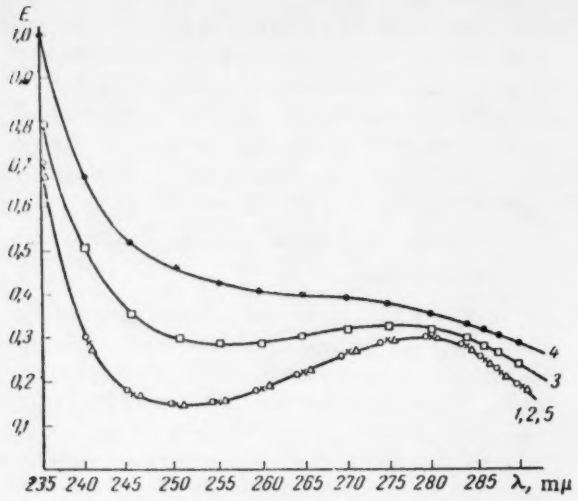
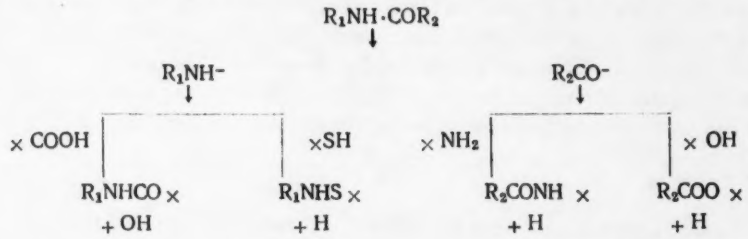


Fig. 2. Relation of optical density of pepsin to nature of the gas present in the solution at the time of treatment. Enzyme concentration 0.02%. Treatment time, 30 min. Treated in an atmosphere of: 1) helium; 2) hydrogen; 3) argon; 4) air; 5) before ultrasonic treatment.

this effect is not found (Table 2). From this effwe can draw the same conclusions as did Él'piner and Sokol'skaya [12], who studied the oxidation of a number of organic acids which occurred under the action of ultrasonic waves in the presence of inert gases. According to their ideas, different inert gases, especially helium and argon, differed from each other in the effect on the chemical activity of OH radicals which appeared in the cavitation field as a result of splitting a molecule of water. Here, in the presence of argon, in a number of cases there was a strengthened oxidation process; helium was inert in this respect. The problem of the mechanism of this effect was discussed in the paper of Él'piner [5].

Thus, the decrease molecular weight of serum albumin and proteolytic enzymes in the presence of oxygen and OH radicals evidently depends on rupture of peptide bonds with formation of molecular fragments (R_1NH^-, R_2CO^-) as radicals [13]. These radicals in the presence of oxygen or argon are inactivated and converted into stable protein molecules with lower molecular weight. In the absence of these gases, but in the presence of hydrogen in the treated solutions, the proteins radicals apparently react with each other or with remaining protein molecules. It is possible that here there occurs the same reaction between protein radicals as was described by Rideal and Roberts [14], who obtained analogous results in the action of ultraviolet rays on protein solutions in the presence of nitrogen. According to their ideas, each of the formed protein radicals can react with OH, COOH, SH, or NH_2 groups of the remaining protein molecules according to the following scheme:



If we start from our data on the effect of ultrasonic waves on enzymes in the presence of hydrogen, we can say that the rupture of peptide bonds with later combination of the protein radicals with protein molecules is not accompanied by loss of enzymatic activity in the enzymes which we studied. Loss of enzyme activity occurs when is splitting of cyclic groups which permits reaction of the protein with active oxygen or OH radicals. As to rupture of the peptide bonds, there is reason to believe that this occurs as a result of the development of mechanical forces in the field of the ultrasonic waves and is independent of what gas saturates the solution under study.

SUMMARY

In an ultrasonic field, proteins (serum albumin, pepsin, and trypsin) in the presence of oxygen in the treated solution split into smaller molecular fragments. In enzymes, such splitting is accompanied by loss in enzyme activity.

The replacement of air by hydrogen in the treated protein solutions leads to increased molecular weight of the proteins. In this study, the proteolytic enzymes do not lose their enzymatic activity. When protein solutions are treated in the presence of oxygen or argon, there is a decrease in absorption maximum in the portion of the

ultraviolet specific for proteins. Spectrophotometric changes in protein structure are not seen when they are treated in water saturated with hydrogen.

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THE ULTRAVIOLET FLUORESCENCE OF GUANINE ON A PAPER CHROMATOGRAM AND IN SOLUTION

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It is known that the paper chromatograms with guanine and substances which contain this base have an intense fluorescence in the blue-violet part of the spectrum. Such a fluorescence of guanine can be observed when the chromatogram from the five purine and pyrimidine bases is inspected in the chemiscope. Adenine and also the pyrimidine bases (cytosine, thymine, and uracil) do not have this luminescence.

The purpose of the present work is to show the presence of fluorescence in purine and pyrimidine bases on chromatograms, to obtain from it the fluorescence spectrum of those which fluoresce in the visible or ultraviolet and to compare these spectra with the fluorescence spectra of the same substances in solution.

METHOD S

Photography of the fluorescence spectra was carried out using an ultraviolet microscope connected with a spectrographic apparatus constructed by Brumberg [1-4]. The fluorescence spectra were photographed: 1) for spots of the purine and pyrimidine bases on the paper chromatograph; 2) for guanine solutions in a microcuvette; 3) for guanine spots obtained after depositing solutions with different pH values on the filter paper. For this purpose we first prepared guanine solutions with pH 1, 3, 5, 7, 9, and 14.

The acid solutions were obtained by adding HCl, the alkaline, with NaOH. To obtain neutral solutions we used a phosphate buffer. A drop of each of the prepared solutions was placed on the filter paper which was placed between a cover glass and a quartz microscope slide, and the fluorescence spectrum was then photographed. In each case the fluorescence spectrum was photographed with the same exposure for the pure solvent.

Since the intensity of fluorescence of guanine adsorbed on paper at optimum pH and concentration is considerably greater than in solution, for the study of effect of pH on intensity and position of the maxima in the fluorescence spectrum the guanine solution was placed on filter paper.

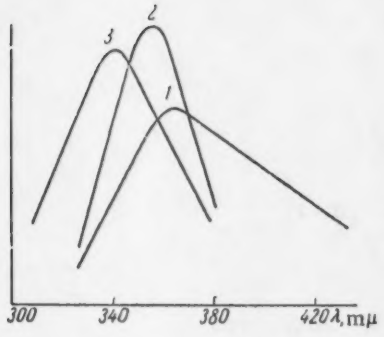
Fluorescence in all the experiments was produced with ultraviolet light with λ= 250-270 mμ, isolated by a chlorine-bromine gas light-filter.

The experiments were carried out on Leningrad chromatographic paper No. 2 (rapid) which had practically no fluorescence in ultraviolet at wave lengths less than 380 mμ. Therefore in constructing the energy distribution curve in the fluorescence spectrum of guanine the fluorescence of the chromatogram paper was not considered (in studying substances with maximum fluorescence in visible light it is necessary to correct for the fluorescence of the paper).

Construction of the energy distribution curve in the fluorescence spectra of the substances studied was carried out by the known methods of photographic spectrophotometry [1-4].

RESULTS

In the study of chromatograms from five purine and pyrimidine bases it was established that the maximum fluorescence of guanine occurred in the ultraviolet portion of the spectrum at λ = 252 mμ. Adenine, uracil, cytosine, and thymine in an exposure even two three times greater than that used for photographing the fluorescence spectrum of guanine gave absolutely no darkening on the plate, that is, under these conditions they did not fluoresce.



Energy distribution curve in the fluorescence spectrum of guanine depending on conditions. 1) Guanine in solution; 2 and 3) guanine adsorbed on filter paper respectively from acid alkaline solutions. The curve does not give the actual relative intensities of guanine fluorescence on filter paper and in solution.

The solution of guanine in 1 N HCl has a fluorescence with a maximum brightness at $\lambda = 365$ mμ which agrees with data given by Duggan et al. [5]. Thus the fluorescence maximum of guanine in solution is shifted as compared with the maximum on the chromatogram by 13 mμ toward the long wave side.

Photography of the fluorescence of guanine spots on filter paper when solutions with different pH were used permits us to draw the following conclusions. In a strongly acid medium (pH 1) guanine has the clearest fluorescence with maximum brightness at $\lambda = 352$ mμ, that is, the fluorescence maximum of the guanine spot when the pure substance is deposited from acid solution into the paper agrees with the maximum in the fluorescence spectrum of guanine in chromatograms also obtained from acid solutions of substances. When the acidity of the solutions is decreased (pH 3,5) the intensity of fluorescence falls considerably and the position of the maximum in the fluorescence spectrum is not changed. Such a decrease in intensity can be explained chiefly by the considerable lessening of the solubility of guanine in weakly acid and neutral solutions. In a strongly alkaline medium, for example, in 1 N NaOH (pH 14), in which the solubility of guanine is quite appreciable, its fluorescence increases. Maximum brightness is found at $\lambda = 343$ mμ, that is, the fluorescence spectrum of the alkaline solution is shifted by 9 mμ to the short wave side compared to the fluorescence spectrum of the acid solution. This shift can be explained by a difference in fluorescence of the hydrochloride salt formed by guanine in the acid medium and the sodium salt formed when it dissolves in an alkaline medium. It is also possible that this shift in the fluorescence spectrum is connected with the formation of a tautomeric form of guanine (oxo form and hydroxy form) in acid and alkaline media, with differing fluorescences.

In the figure we give the energy distribution curve in the fluorescence spectrum of the acid solution of guanine (1); of guanine adsorbed on filter paper from acid (2) and from alkaline (3) solutions.

SUMMARY

We have studied the distribution of energy in the fluorescence spectrum of guanine in chromatograms, in solution, and of guanine adsorbed on filter paper from acid and alkaline solutions. The position of the maximum in the guanine fluorescence spectrum in acid and alkaline media is different.

The slight intensity of fluorescence of the paper used for chromatography especially in the ultraviolet region, and the considerably increased clarity of the fluorescence of many substances when they are adsorbed on filter paper, as compared to solutions of the same substance, permit study of the fluorescence spectra of different substances directly on the chromatogram. This process of obtaining the spectra can also be used in studyng the fluorescence of pure substances.

In conclusion we express our thanks to E. M. Brumberg for directing this work and to T. N. Evreinova and R. G. Broun for advice and supply of the preparations.

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SOME PROPERTIES OF MITOCHONDRIAL MONOAMINO OXIDASE OF RAT LIVER AND BRAIN

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Monoamino oxidase (MO) (tyraminase or amino oxidase) plays an important part in the organisms of humans and animals, carrying out oxidative deaminations and also inactivating a number of biogenic amines (serotonin, noradrenaline and others) which have important physiological, and under some conditions, pathological actions [1-4]. MO is very strongly bound to mitochondrial structures [3,4]; it has not yet been possible to obtain soluble preparation of this enzyme and to purify it. The so-called "soluble amino oxidase" [5] obtained by fractionation of guinea pig liver homogenates by preparative ultracentrifuging is much less active than the structurally bound MO.

MO, in distinction to diamino oxidase, evidently does not require for its action the presence of pyridoxal phosphate. The nature of the active groups of MO and the mechanism of its action are not known; it is thought that it contains sulfhydryl groups and a flavin coenzyme [4].

Abbreviations used: MO – monoamino oxidase; HQ – 8-hydroxyquinoline; DEC – sodium diethyldithiocarbamate; CH – cyclohexanediamine tetraacetic acid; OP – o-phenanthroline.

Preparations: OP, dimethylglyoxime, plumbone IREA, trihydroxyglutaric acid, nitroso-R-salt were kindly supplied to us by M.Z. Portashnikova, I. G. Shafran and N. M. Dyatlova (IREA); hexamethylenediamine tetraacetate, benzhydrylamine diacetate, by Prof. R. P. Lastovskii (IREA); cyclohexanediamine tetraacetic acid by V. G. Yashunskii (VNIKhFI), for which we express our thanks.

In the course of our work we obtained new results which show the possible role of metals in the action of the structurally bound MO of mitochondria.

METHODS

In this study we used male white rats weighing 140-160 g, fed an ordinary mixed diet. The animals were killed by decapitation; the liver tissue and in some experiments (Table 1) the brain were rapidly homogenized with cooling in a glass Potter-Elvejhem homogenizer with 9 times the quantity of 0.1 M phosphate buffer [6], pH 7.4 which contained 0.01 M versene [7]. The resulting homogenate was centrifuged at 1000 rpm for 5 min to remove cell fragments and nuclei. The mitochondria were precipitated by centrifuging the supernatant liquid at 10,000 rpm for 10 min. The precipitate of mitochondria was washed on the centrifuge with the buffer solution, in the smallest possible volume of this solution, and was dried in the frozen state. The weight of dried mitochondria preparation obtained from 10 g of fresh liver tissue and brain of rats averaged 0.68 and 0.23 g; the enzyme activity of the preparations did not decrease when they were kept for 2-3 months at 2°.

Extraction of the lyophilized mitochondria with acetone was carried out in the cold (at –10°); treatment by other organic solvents (isooctane, heptane, butanol, toluene) was at room temperature. Extraction of the aqueous salt suspension of the mitochondria by isooctane or heptane was carried out by the method of Nason and Lehman [8]. For removal of traces of organic solvents from the enzyme preparations we submitted them to lyophilic drying.

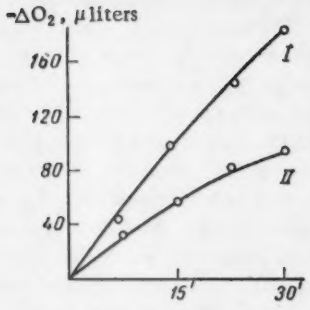

Fig. 1. Effect of phenazine methyl sulfate on the activity of rat liver monoamino oxidase. Composition of the sample – final concentration $8.65 \cdot 10^{-3}$M, fresh mitochondria in an amount equivalent to 0.5 g of fresh liver. The remaining components of the sample and conditions of the experiment are described in the "Methods" section. I) Absorption of oxygen in the absence of phenazine methyl sulfate; II) the same in the presence of $1.7 \cdot 10^{-8}$M phenazine methyl sulfate (final concentration).

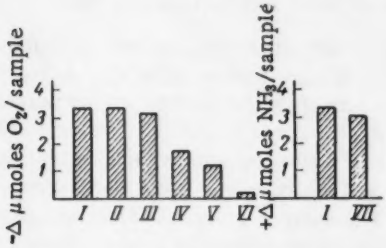

Fig. 2. Effect of extraction with some organic solvents on MO activity of rat liver mitochondria. Sample contained 10 mg (6.8 mg protein) of lyophilized mitochondria from rat liver. I) Nonextracted mitochondria; II) mitochondria after extraction with isooctane; III) mitochondria after extraction with heptane; IV) mitochondria after extraction with butanol; V) mitochondria after extraction with toluene; VI) mitochondria after extraction with a mixture of equal volumes of ethyl alcohol and ether; VII) mitochondria after extraction with acetone.

MO activity was measured by the amount of amino nitrogen liberated in the incubation of a neutralized water solution of tyramine (1 mg or 5.8 μmoles in a sample) in the presence of the mitochondria preparation (10 mg or 6.8 mg of protein in the sample) for 1.5 hours at pH 7.4 (0.05 M barbiturate buffer) in an atmosphere of oxygen at 37.5° and 80-100 oscillations per min in a Warburg apparatus. For fixation of the sample and precipitation of the protein at the end of incubation, we added TCA (final concentration 5%). The total volume of the sample was 2 ml. The precipitate was separated by centrifuging and the content of ammonia in the supernatant liquid was determined by isothermal distillation according to Conway with later nesslerization. Under these conditions the amount of ammonia liberated by MO of liver and brain mitochondria corresponded respectively to 4.8 and 2.5 μmoles per sample (or 0.47 and 0.24 μmoles/hour·mg of protein).

Absorption of oxygen was determined manometrically according to Warburg [6]. In the main Warburg vessel we placed the enzyme solution, in the side tube the substrate solution in the amounts given above. The central inset contained 0.2 ml of 1 M KOH. The final volume of the reaction mixture was 2 ml; the gas phase was oxygen.

In the experiments with rat liver mitochondria the oxygen absorption averaged 0.25 μmoles / hour·mg of protein.

The experiments with carbon monoxide were carried out in a blackened vessel [9]; the gas phase in these experiments consisted of 95% nitrogen and 5% CO (in the control samples, 95% nitrogen and 5% oxygen).

The water solutions of complex forming reagents were prepared immediately before the experiment and were carefully neutralized. The metal ions were added to the samples as sulfates.

RESULTS OF THE STUDY

In the study of the active groups and mechanism of MO action we tried to dissolve this enzyme, using as a hydrogen acceptor the dye phenazine methyl sulfate which was used successfully [10,11] for replacing the cytochrome system in the extraction of other structurally bound oxidizing enzymes from mitochondria (succinic dehydrogenase, choline dehydrogenase). It was shown that phenazine methyl sulfate, which in our experiments acted as an active hydrogen transporter in the succinic dehydrogenase system, could not serve as an artificial acceptor in the oxidation of tyramine under the influence of MO, and hindered appreciably the action of this enzyme (Fig. 1). These results agree with the fact that MO of mitochondria, in distinction to succinic and choline dehydrogenases, apparently reacts with oxygen without participation of the cytochrome system [3,4]. MO is relatively stable when the lyophilized mitochondria are treated with some organic solvents – isooctane heptane, acetone (in the latter case it is important to cool carefully during the experiment).

In Fig. 2 we also see that some other organic solvents inactivate MO to a greater or less degree. Repeated freezing of the mitochondrial suspension does not change MO activity. After thawing of the mitochondrial suspension,

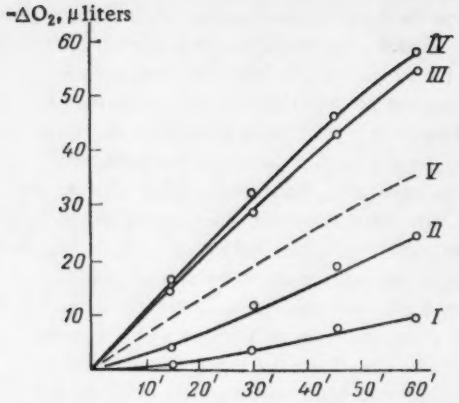

Fig. 3. Monoamino oxidase activity of fractions of decomposed rat liver mitochondria. 1% suspension of lyophilized rat liver mitochondria in 0.1M phosphate buffer (pH 7.4) submitted to two freezings and thawings followed by centrifuging at 10,000 rpm for 15 min; the precipitate again suspended in the same buffer. Samples: I) tyramine + supernatant liquid fraction; II) tyramine + precipitate fraction; III) tyramine + mixture of supernatant liquid and precipitate; IV) tyramine + initial suspension of mitochondria decomposed by freezing; V) arithmetical sum of I and II.

it is possible by centrifuging in 0.1 M phosphate buffer (pH 7.4) at 10,000 rpm for 15 min to separate a soluble, thermolabile fraction from the insoluble residue of mitochondria, the addition of which increases the uptake of oxygen in the oxidation of tyramine under the influence of MO which in this treatment remains in the precipitated fraction (Fig. 3). However, the addition of the soluble factor does not increase the amount of ammonia formed from tyramine (Fig. 4). In the presence of $1 \cdot 10^{-2}$M semicarbazide, the stimulating action of the soluble fraction obtained under the conditions described above on the activity of the MO of the insoluble mitochondrial residue is no longer shown (Fig. 4). Evidently, under these conditions it is not a component of MO which dissolves, but an aldehyde oxidase which oxidizes the product of the action of the amino oxidase, an aldehyde, into the corresponding acid [3,4].

We have not found any report of the separation of the structurally bound MO of mitochondria from an aldehyde oxidase. The separation of the aldehyde oxidases from "soluble" amino oxidase of guinea pig liver [5] was reported by centrifuging a homogenate in the preparative ultracentrifuge. The activity of rat liver and brain mitochondria, as Table 1 shows, is suppressed by a number of compounds which form chelation complexes with metals. Destruction of the mitochondria by repeated freezing and thawing to increase their permeability has no effect on the suppressing action of HQ and DEC.

Inhibition of the acidity of MO by a number of complex-forming compounds (HQ, DEC, plumbone IREA, OP) is reversible: the enzyme activity of the preparation is fully restored by washing out the inhibitor and even by ordinary dilution of the sample. Repeated addition of HQ to the enzyme whose activity has been restored by washing the mitochondria suspension after incubation with HQ again produces inhibition of MO. The inhibiting action of HQ on MO activity is not removed by addition of a fivefold excess of cysteine or glutathione (with respect to the concentration of inhibitor). The addition of ions of several divalent metals to the enzyme preparation, first incubated with HQ or CH, partly removes the inhibiting action of these complex formers on MO (Table 2). In experiments with HQ, Zn^{++} is most effective, in experiments with CH, Cu^{++}.

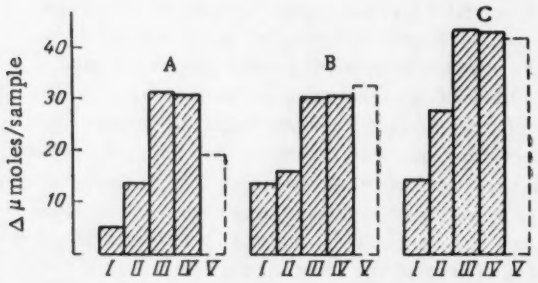

Fig. 4. Effect of soluble mitochondria fraction from rat livers on activity of MO precipitate. Sample contained 10 mg (6.8 mg protein) of lyophilized mitochondria or the corresponding fraction. Designation of samples the same as in Fig. 3. A) Oxygen absorption; B) the same in the presence of $1 \cdot 10^{-2}$M semicarbazide; C) liberated ammonia.

Increased concentration of metal ions does not cause fuller removal of inhibiting action of the complex forming reagents. Excess of metal ions causes inhibition of MO; in the absence of complex-forming compounds the inhibiting action of metals appears even at rather low concentrations (Table 3). According to the literature data [12], with further decrease in concentration of metal salts (on the order of 1-2 times) they show a specific stimulating action on the activity of rat liver MO. Preliminary addition to HQ of metal salts in amounts required for complete binding by these complex formers [13] prevents their inhibiting action on MO.

In experiments with DEC and OP we did not find conditions in which ions of zinc, copper, or iron could decrease the action of these inhibitors on MO. Carbon monoxide in the dark under conditions

TABLE 1

Effect of Complex Forming Reagents on the MO Activity of Rat Liver and Brain Mitochondria

Reagent	Concentration (M)	Inhibition of NH_3 liberation (in %, average values)	
		liver	brain
8-Hydroxyquinoline	$3 \cdot 10^{-4}$	6	—
"	$1 \cdot 10^{-3}$	51	—
"	$2 \cdot 10^{-3}$	67	82
"	$4 \cdot 10^{-3}$	79	—
Plumbone IREA	$1 \cdot 10^{-1}$	61	—
Diethyldithiocarbamate	$1 \cdot 10^{-3}$	32	36
"	$2 \cdot 10^{-3}$	38	—
"	$1 \cdot 10^{-2}$	42	—
o-Phenanthroline	$2 \cdot 10^{-3}$	27	50
αα'-Dipyridyl	$1 \cdot 10^{-3}$	26	—
DL-Trihydroxyglutaric acid	$1 \cdot 10^{-1}$	12	—
Nitroso-R-salt	$1 \cdot 10^{-3}$	10	—
Cyclohexanediamine tetraacetic acid	$5 \cdot 10^{-3}$	46	71
Ethylenediamine tetraacetic acid (versene)	$1 \cdot 10^{-2}$	28	57
Hexamethylenediamine tetraacetate	$1 \cdot 10^{-3}$	9	—
Benzydryl amine diacetate	$1 \cdot 10^{-3}$	0	—
Benzylhydryl amine diacetate	$1 \cdot 10^{-3}$	0	—
Sodium pyrophosphate	$1 \cdot 10^{-3}$	0	—
Parafuchsine hexaacetate	$1 \cdot 10^{-3}$	0	—
Dimethylglyoxime	$1 \cdot 10^{-3}$	0	—
Thiourea	$1 \cdot 10^{-3}$	0	—

TABLE 2

Effect of Metal Ions on Inhibition of MO from Rat Liver Mitochondria by 8-Hydroxyquinoline and Cyclohexanediamine tetraacetate. The figures denote percent decrease in liberated NH_3 compared to the control sample (without comples former and cations). The samples contain $2 \cdot 10^{-3}$M HQ or $5 \cdot 10^{-3}$M CH. Metals added in concentration of $1 \cdot 10^{-3}$M (in the experiments with CH) in the form of sulfates after 30 min incubation of the enzyme at room temperature with the complex former.

Metal ion	HQ	CH
Not added	67	46
Zn^{++}	20	30
Mn^{++}	39	—
Fe^{++}	40	—
Cu^{++}	59,5	28
Co^{++}	41	36
Cd^{++}	47,5	—
Mg^{++}	67	38

described in the "Methods" section, did not affect the MO activity of rat liver mitochondria.

Long dialysis in an apparatus for rapid dialysis [14] using a suspension of destroyed mitochondria against distilled water or against a water suspension of cationite KU-2 (250 mesh) in the sodium form gave no appreciable decrease in MO activity.

DISCUSSION OF RESULTS

In the literature we found very little information on the action of complex-forming compounds on MO. There are indications of the inhibiting action of sodium azide on MO of plant and animal tissues [15]. According to unpublished data of Lagnado, cited in the review of Sourkes [3], OP at concentrations of $1 \cdot 10^{-3}$ inhibits the MO of animal tissues; DEC inhibits MO only for plants, but not in animal tissues.

As our data show, the activity of MO from rat liver and brain mitochondria is inhibited by the action of substances with different chemical structures which possess only one common property, the ability to form complex compounds with ions of di- and polyvalent metals. In a number of cases we showed the possibility of partly removing the inhibiting action of complex forming compounds on MO by adding the salts of metals, and also of preventing this action by preliminary binding of the complex formers with metals. These facts permit us to conclude that inhibition of MO by the reagents studied depends on the ability of these compounds to bind one or another metal which is needed for the action of the enzyme.

We can assume that the inhibiting action of some complex-forming compounds occurs by their action on constituent SH groups in the MO protein molecule. However, in our experiments the addition to the samples of a

TABLE 3

Effect of Metal Ions on MO Activity of Rat Liver Mitochondria. Figures express inhibition of NH_3 liberation in samples with metal ions in percent compared to control samples (without addition of metal salts)

Concentration (M)	Zn^{++}	Fe^{++}	Co^{++}	Mg^{++}	Cu^{++}	Mn^{++}
$1 \cdot 10^{-4}$	7	—	—	—	12	—
$2 \cdot 10^{-4}$	9	9	0	—	—	—
$5 \cdot 10^{-4}$	30	—	—	—	—	—
$1 \cdot 10^{-3}$	36,5	34	12	—	—	0
$5 \cdot 10^{-3}$	67	—	—	—	—	15
$2 \cdot 10^{-2}$	—	—	—	0	—	—

considerable excess of cysteine or glutathione did not decrease the inhibiting action of HQ on MO, which contradicts this assumption.

Our results do not furnish a basis for a conclusion as to the specific role of any definite metal in the action of MO or as to the concrete function of a metal. The more obvious ability of Zn and Cu ions as compared to ions of other divalent metals to decrease the inhibition of MO caused by HQ and CH evidently depends on the ability of these metals to give more stable complexes with the complex-forming compounds [16-18]. It is possible that mitochondrial MO belongs to the group of enzymes whose activity and stability requires formation of a dissociative complex with cations of one of several (mutually replaceable) metals which do not take part directly in catalyzing the enzyme reaction [19,20,22]. However, the results of the above described experiments also agree with the assumption that MO has the properties of a "true" metalloenzyme. Enzymes of this type are characterized as follows: 1) metals, as a rule, take a specific part in the enzymatic reaction; 2) metals are firmly bound in stoichiometric ratios with the protein component and can be separated only with difficulty by dialysis or other methods; 3) the inhibiting action of complex-forming compounds is reversible when they are removed or by the addition of the salts of the metals of the enzyme itself or of other cations which can combine with the inhibitors. For a study of the mechanism of the inhibition effect of the complex-forming compounds and for a solution of the question of the role of metals in the action of MO we must obtain this enzyme in purified form.

Lagnado and Sourkes [23] in a study of the enzymatic reduction of tetrazole derivatives by rat liver tissue preparations (homogenates, mitochondria) in the presence of tyramine noted an inhibiting action on this process by cyanide, azide, OP and α,α'-dipyridyl in high concentrations ($1 \cdot 10^{-2}$M). As is known, cyanide does not have an inhibiting action on MO [4]. It is probable that in the experiments of these authors the inhibition of reduction of tetrazole was due to the effect of inhibitors not only of MO, but also of other enzymes in the system. On the basis of our results, we can assume that the action of OP and α,α'-dipyridyl in this complex system is directed exclusively to MO.

SUMMARY

The MO activity of rat liver and brain mitochondria is reversibly inhibited in vitro by a number of complex-forming reagents (8-hydroxyquinoline, plumbone, diethyldithiocarbamate, and others).

Partial removal of the inhibition caused by 8-hydroxyquinoline and cyclohexanediamine tetraacetate occurs on addition to the sample of ions of some divalent metals, especially the ions of Zn and Cu. Incubation of the complex-forming reagents with metal salts before addition to the enzyme system prevents their inhibiting action on the activity of MO.

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THE EFFECT OF CHLORTETRACYCLINE ON THE ENZYMATIC HYDROLYSIS OF ADENOSINE TRIPHOSPHORIC ACID

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In connection with the wide use in chemotherapy of antibiotics of the group of the tetracyclines, there is naturally great interest in the question of the mechanism of their action both on bacterial cells and on the tissues of animal organisms.

The ability of this group of antibiotics to form chelation complexes with metals is a reason for studying the problem of whether the basis of the action of these antibiotics does not lie in the formation of complexes with divalent cations, the activators of different enzyme systems.

In favor of this possibility are the numerous findings of the ability of cations to hinder or fully prevent the action of tetracyclines on the growth of bacterial cells and on separate enzyme systems of both bacterial and animal origin [1]. According to the ideas of Albert [2], the toxic action of antibiotics which can form chelation complexes with metals does not depend on their binding these metals, but on the toxicity of the complexes themselves.

Our attention was attracted to the question of the action of the effect of chlortetracycline on the enzymatic hydrolysis of ATP, a process, as is known, which plays an important part in muscular work and cell function. Since this process depends on ions of potassium, magnesium, and calcium, it is altered by the effect of a number of complex forming compounds, and it seemed interesting to us to compare the action of chlortetracycline with the action of the widely used complex former, versene (EDTA). Therefore we carried out parallel investigations under the same conditions for both "poisons".

In the present communication we give data concerning the effect of these substances on the hydrolysis of ATP by water extracts and preparations of myosin from rat cardiac muscle.

EXPERIMENTAL PART

The experiments were carried out on white rats of weight 100-150 g. The animals were decapitated, the hearts were quickly removed and freed from blood with filter paper. The heart was minced in the cold and further homogenized in a glass homogenizer.

We estimated the enzymatic splitting of phosphorus from ATP by difference in increase in inorganic phosphorus between samples incubated with the enzyme and without it, in which it was added after fixation by TCA. Under the conditions of our experiment the liberation of inorganic phosphorus from the enzyme preparation during incubation was negligible. Inorganic phosphorus was determined by the method of Weil-Malherbe and Green [3] in a butanol extract from the sample. Since in the samples which contained chlortetracycline after reduction, we obtained not a blue, but a green color, we were always forced to carry out the determination of phosphorus in the presence of a corresponding concentration of the antibiotic, and to obtain a standard curve under these conditions. For this purpose, after fixation of the samples we equalized the concentration of antibiotic in them. The application of this method did not permit us to use very high concentrations of chlortetracycline since the latter in and acid medium gives cloudiness and even a precipitate. However, if not more than 100 mg of chlortetracycline is contained in the test tube during the determination, cloudiness does not occur, and the use of the method

is entirely feasible. All the reagents were prepared in twice-distilled water, distilled in a glass apparatus. We used chlortetracycline hydrochloride in this work. Chlortetracycline, versene, and ATP were disolved in water and neutralized with NaOH before the experiments. The preparation of the enzymes and the composition of the samples are described separately for each series of experiments.

Effect of Chlortetracycline on Water Soluble ATP-ase of Rat Cardiac Muscle

In these experiments we tried to find the conditions most suitable for explaining the action of chlortetracycline.

The concentration of chlortetracycline was higher than the concentrations of ATP. The basis for this is the observation of Santi and Bruni [4] who showed that the action of tetracyclines on the growth of bacterial cells decreased ATP, and who assumed a direct reaction of ATP with the antibiotics. If this reaction actually occurs, there may be danger that the antibiotic can be neutralized by excess substrate. From this consideration, we used a concentration of ATP of $2 \cdot 10^{-4}$M in our preliminary experiments.

According to the data of Brody et al. [5], some enzymes, such as hexokinase, are suppressed by tetracyclines only when they are present in small amounts. Large quantities of these enzymes, in the opinion of the authors, can complete successfully with the tetracyclines for ion activators. Because of this possibility we used very dilute enzyme preparation.

We homogenized 200 mg of minced rat heart in 10 ml of twice distilled water; the homogenate was centrifuged and the centrifugate was further diluted 25 times.

The complete sample contained: 0.5 ml of acetate-veronal buffer with pH 7.0, 0.5 ml of antibiotic solution, 0.5 ml of enzyme solution, 0.5 ml of 10^{-3}M $MgCl_2$, and 0.5 ml of 10^{-3}M ATP. We showed by special experiments that the use of an ordinary borate buffer is unsatisfactory since it neutralizes the action of chlortetracycline, which occurs well in veronal and acetate buffers. This may be explained by the formation of a complex compound of chlortetracycline with the anion of boric acid which has been described in the literature [6].

The total volume of incubated solution was 2.5 ml. In some experiments the volume of a lacking ingredient was replaced with twice-distilled water. Incubation was carried out for 15 min at 37°. After incubation, the sample was cooled and fixed by addition of 0.5 ml of 50% TCA. Also, in the samples we equalized the chlortetracycline concentrations and immediately transferred 2 ml of samples into butanol with molybdate.

The average data from a series of experiments (Fig. 1) show the effect of chlortetracycline and versene on the intensity of hydrolysis of ATP in water extracts of rat cardiac muscle. The activity of the process is expressed in percent. For 100% activity we took the amount of inorganic phosphorus liberated from ATP in the sample which contained no "poisons", with all other conditions the same.

It is apparant from the results given in Fig. 1A that without addition of magnesium, which is an activator for water-soluble ATP-ase, suppression of the process occurs only at concentrations of chlortetracycline surpassing $1 \cdot 10^{-4}$M; at a concentration of $4 \cdot 10^{-4}$M 60% inhibition is attained. Versene in the same concentrations suppresses the process much more strongly.

The results of the experiments (Fig. 1B) run in the presence of $MgCl_2$ at a concentration of $2 \cdot 10^{-4}$M showed that under these conditions a low concentration of both chlortetracycline and versene can even stimulate the process. The amount of stimulation decreases with increasing concentration of "poison" and goes over to inhibition. As in the previous experiments activation and inhibition by versene are shown more clearly. The stimulating action of low concentratios which we observed is easiest to explain by the presence of excess Mg^{++} ions and the fact that the complex forming compounds (chlortetracycline and versene) which bind the excess of this ion bring its concentration to the optimum. To test this idea, in the next series of experiments we varied the magnesium concentration while keeping the concentration of "poisons" constant.

In Fig. 1C we give average results of this series of experiments. For 100% activity of the process we took the activity in samples which did not contain either $MgCl_2$ or "poisons". Curve 1 characterizes the activity of the process depending on $MgCl_2$ concentration. The maximum activity under the conditions of our experiments is reached at $MgCl_2$ concentration of $1 \cdot 10^{-4}$M, that is, at a ratio of ATP to Mg^{++} of 2:1; with increasing Mg^{++} concentration the activity diminishes. However, the peak of the curve at concentration $1 \cdot 10^{-4}$M is small. Chlortetracycline at a concentration of $1 \cdot 10^{-4}$M increases the activity of the process at Mg^{++} concentrations below the

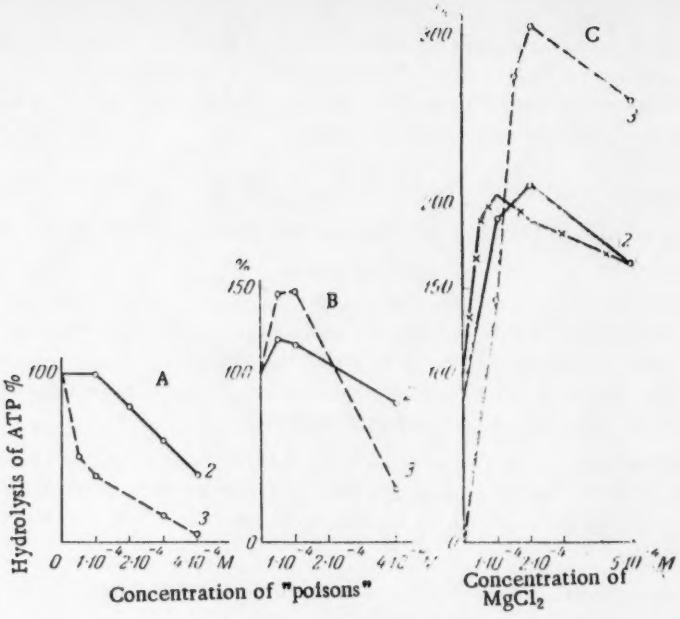

Fig. 1. Effect of chlortetracycline and versene on the intensity of hydrolysis of ATP in water extracts of rat cardiac muscle. A) Without activator; B) in the presence of $MgCl_2$ $2 \cdot 10^{-4}$M; C) "poisons" at concentration of $1 \cdot 10^{-4}$M. 1) Control; 2) with chlortetracycline; 3) with versene.

optimal (curve 2) or equal to it. At Mg^{++} concentrations above optimal, chlortetracycline somewhat stimulates the process. The stimulation of the activity of the process is then about the same as is found at the optimal Mg^{++} concentration in the absence of "poisons". Thus, if the effect of chlortetracycline on enzymatic splitting of ATP in water extracts of cardiac muscle can be fully explained from our point of view by the complex forming properties of the antibiotic, then the effect of versene on this process cannot be explained solely by this.

The ability of versene to activate ATP-ase under definite conditions is already well known. Thus, Gross [7] found that hydrolysis of ATP in cardiac muscle homogenates with activation by Ca^{++} ion is suppressed by high concentrations of versene and activated by small ones. A number of investigators (Friess [8], Bowen et al. [9]) have observed that versene activates myosin ATP-ase at high concentrations of KCl in the medium. However, these findings have not yet been explained in a saytisfactory manner.

Effect of Chlortetracycline on Splitting of ATP by a Myosin Preparation

The myosin solution was obtained by the somewhat modified method of Lyubimova and Pevzner [10]. A mince of 200 mg of rat heart was homogenized in 10 ml of twice-distilled water, the residue was separated by centrifuging and washed once more with the same volume of twice-distilled water. The precipitate was then mixed energetically for 10 min with 5 ml of 0.6 M KCl in a bicarbonate buffer, and in the experiments we used the supernatant liquid after centrifuging. The whole operation was carried out in the cold. The experiments of this series were carried out in a bicarbonate buffer with pH 8.2. $CaCl_2$ was the activator for the ATPase. The volume of the sample was the same as in the previous experiments. A volume of 0.75 ml of buffer was added and 0.25 ml of enzyme solution; the concentrations of the initial solutions of ATP, antibiotic, and activator were varied. Incubation at 27° was carried out for 7 min. In this series of experiments, as in all the following ones, we used a concentration of $1 \cdot 10^{-3}$M ATP, since we showed by special experiments that the direct reaction of ATP with chlortetracycline and assumed by Santi and Bruni [4] did not occur under our conditions.

The resulting data are shown in Fig. 2A. For 100% activity we took the activity of hydrolysis of ATP in samples without addition of activator or "poisons". As the figure shows, the activity of the process in samples without the addition of Ca^{++} is almost not suppressed by chlortetracycline (curve I). In the presence of Ca^{++} ion at a

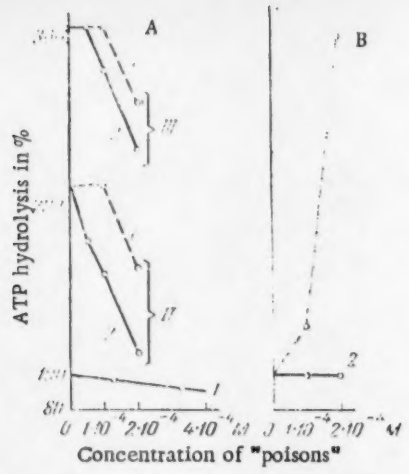

Fig. 2. Effect of chlortetracycline and versene on the intensity of hydrolysis of ATP by preparations of myosin from rat heart muscle. A: I) without Ca^{++}; II) Ca^{++} $5 \cdot 10^{-4}$M ; III) Ca^{++} $1 \cdot 10^{-3}$M ; B) without Ca, in 0.6 M KCl. 1) With versene; 2) with chlortetracycline.

concentration of $5 \cdot 10^{-4}$M (curve II), and at a Ca^{++} concentration of $1 \cdot 10^{-3}$M (curve III), chlortetracycline inhibits the process much more strongly than does versene. Thus, in the study of myosin ATP-ase the picture is the reverse of that found when using water soluble ATP-ase. For myosin ATP-ase, chlortetracycline is a stronger "poison" than is versene. It is possible that this is connected with a greater affinity of chlortetracycline for Ca^{++} than for Mg^{++}. However, we have no evidence for this assumption.

It is interesting to determine whether chlortetracycline can, like versene, activate myosin ATP-ase in a system which contains a high concentration of KCl [8]. For this purpose we added KCl to all the samples to a final concentration of 0.6 M. Calcium was not added to the samples. As Fig. 2 B shows, the activating effect of versene is well reproduced, while chlortetracycline does not show a similar effect. Thus, when chlortetracycline acts on the enzymatic hydrolysis of ATP we do not find a paradoxical effect such as is seen with versene.

The inhibition of ATP hydrolysis by chlortetracycline in water extracts of cardiac muscle and in myosin solutions is evidently connected with binding of the activating ions. If we assume that inhibition of the process by chlortetracycline is related to its formation of a toxic complex with any metal, then we must agree that Ca^{++} and Mg^{++} ions, added in excess, can easily force out the metals from this complex. Since in the blood there are more than enough ions for "neutralization" of the therapeutic dose of chlortetracycline, the data of our work do not supply a basis for assuming that the use of chlortetracycline in chemotherapy can lead to a marked change in the hydrolysis of ATP in cardiac muscle.

SUMMARY

The effect of chlortetracycline, and also of versene, on the hydrolysis of ATP in water extracts and in solutions of myosin from cardiac muscle of rats depends on the concentration of the activating ions Mg^{++} and Ca^{++}.

At low and optimal concentrations of Mg^{++} both of these preparations have an inhibiting effect on the hydrolysis of ATP in water extracts. Chlortetracycline then inhibits hydrolysis more weakly than does versene. At concentrations of Mg^{++} greater than the optimal, chlortetracycline and versene activate the process. However, the intensity of the process activated by chlortetracycline then does not surpass its intensity at the optimun Mg^{++} concentration in the absence of the antibiotic, and the intensity of the process activated by versene considerably surpasses the corresponding level. When activation is by Ca^{++} ions, chlortetracycline is stronger than versene, and can suppress splitting of ATP in myosin solutions. When 0.6 M KCL is contained in the solution, chlortetracycline, in distinctions to versene, does not activate myosin ATP-ase.

Laboratory worker T. F. Shukalova took part in this work for which the authors express their thanks.

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TRANSFORMATIONS OF NICOTINE AND AMINO ACIDS IN THE RIPENING TOBACCO SEED

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Nicotine is formed in the tobacco plant at a very early stage in the sprouting of the seed, at the moment when the germ begins to develop and deep changes occur in the reserve of nutritive substances under the influences of complex enzyme systems. Its formation occurs during the whole developmental period of the plant, and the nicotine content of the seed decreases only during ripening and falls to zero when the seed is fully mature.

When the tobacco seed sprouts there is a relation between the formation of nicotine and the transformations of nitrogenous substances [1]: the quantity of bound and free glutamic acid decreases while the amount of nicotine continuously rises. It has been assumed that this amino acid takes part in nicotine synthesis, and this has been confirmed by experiments carried out by other investigators using labeled compounds [2].

It is of interest to conduct experiments on the transformations of nicotine and amino acids in ripening tobacco seeds, that is, in the period of the final cycle of ontogenesis of the plant.

The theory of Robinson [3] on the synthesis of alkaloids in plants from amino acids is at present very commonly accepted.

The use of isotopes indicates the precursors of nicotine. It has been shown that various amino acids enter its molecule and the position of the labeled atoms of the precursors has been established. The inclusion of the N-methyl group in the nicotine molecule occurs by transmethylation, involving methionine, glycine, and other compounds. The exact mechanism of this reaction and the enzyme systems which take part in it still remain unknown [4-8]. In work with the introduction into the tobacco plant of ornithine and glutamic acid, it has been shown that these compounds enter the pyrrolidine ring of nicotine; the path of their transformations can be followed and the position of the different carbon atoms of the precursors in the ring can be determined [2, 9-12]. The biosynthesis of the pyridine ring of nicotine can occur through nicotinic acid [13].

Starting from the existing data on the participation of several amino acids in the biosynthesis of nicotine, we were interested in following the transformations of the whole complex of nitrogenous substances, especially proteins and free amino acids, in the ripening tobacco seed.

METHODS

For this study we used the seeds of Nicotiana tabacum at two stages: unripe (green pods with white seeds) and ripe (brown pods with brown seeds). The seeds in the air-dried and ground state were extracted with ether and then the proteins were obtained from the material by successive removal with the corresponding solvents.

Albumins was removed by water extraction in the ratio 1: 10 for three hours, three times at a temperature of 5-8° and constant stirring in the presence of toluene. The extract was filtered and precipitated with alcohol in the ratio 1: 2 . Globulin was removed with 5% NaCl solution and was precipitated by electrodialysis at 18 volts, current strength 25 ma for 5 hours at 5-6° . The globulin was obtained in crystalline form. After removal of gluten the sample was treated with 0.5% NaOH, filtered, and the protein precipitated by acidification with weak acetic acid at pH 4.6. The precipitates of the three protein fractions were filtered , washed, and dried lyophilically. The preparations were kept in a desiccator over phosphoric anhydride.

TABLE 1

Content of Amino Acids in Proteins of Unripe and Ripe Tobacco Seeds (in %)

Amino acid	Protein fraction					
	albumin		globulin		gliadin	
	unripe	ripe	unripe	ripe	unripe	ripe
Aspartic	8,0	7,5	7,9	9,8	12,1	7,4
Glutamic	16,9	20,3	15,1	28,0	10,1	22,0
Serine	4,0	3,3.	4,7	4,0	4,0	4,3
Glycine	4,2	4,6	6,8	5,5	8,0	4,0
Threonine	2,3	2,5	4,9	4,8	6,0	5,5
Alanine	2,0	3,5	2,0	4,3	2,1	3,5
Tyrosine	2,0	1,3	1,0	0,7	2,3	1,4
Valine	4,0	3,6	2,8	3,9	4,0	3,7
Methionine	0,4	0,9	0,8	0,7	0,9	1,0
Leucine	5,8	7,2	6,4	8,5	8,0	7,3
Phenyl alanine	4,3	4,4	3,9	4,4	4,5	4,2
Arginine	4,7	9,2	7,0	10,0	7,8	10,1
Histidine	1,7	1,9	2,3	2,6	2,1	2,4
Lysine	3,6	5,2	2,0	3,3	3,1	4,4
Proline	2,2	2,2	2,8	2,2	5,1	5,9
Cystine	2,0	1,9	3,0	3,0	3,0	3,3

Free amino acids were removed from separate samples of tobacco seeds with 70% ethanol with constant stirring for 100 hours. Ethanol and water were distilled off in a vacuum. The dry amino acids were dissolved in 1 ml of distilled water and kept with antiseptic at 0°.

The protein samples, which contained about 1.6 mg of nitrogen, were hydrolyzed with 1 ml of 6 N HCl with boiling for 24 hours. At the end of the hydrolysis, humin substances were filtered off and washed with 2 ml of hot distilled water. The wash water was added to the hydrolyzate. HCl was removed in a vacuum, dessicator over KOH and P_2O_5. The hydrolyzate was diluted with much water and brought to pH 6. Then the hydrolyzate was evaporated dry and dissolved in 1 ml of distilled water. The resulting hydrolyzates were kept with a drop of toluene at 0°. For the determination of amino acids we used the method of quantitative paper chromatography in the modification which we described previously [1]. The determination of nicotine in the seeds was carried out by the method of Pfihl: steam distillation and precipitation of nicotine in the distillate by picric acid [14].

RESULTS

Using a two-phase chromatogram, we could not discover a difference in the qualitative content of free and bound amino acids in unripe and ripe tobacco seeds. The results of the quantitative determination of bound amino acids in the proteins of unripe and ripe seeds are given in Table 1. Analysis of these results shows that the content of the amino acids: serine, threonine, methionine, cystine, proline, and histidine during the ripening of the seeds does not change much. There is a slight decrease in content of aspartic acid (albumin and globulin fractions) and of tyrosine during ripening of the seeds. The amino acids: alanine, arginine, lysine, leucine (albumin and globulin fractions) and especially glutamic acid (in all fractions) accumulate intensively in the ripe seeds.

The data on the content of free amino acids during ripening of tobacco seeds is given in Table 2 and shows that the content of all the free amino acids in the unripe seeds is particularly high, and with ripening it progressively falls due to their use in the synthesis of the reserve proteins of the ripe seeds. This effect is in good agreement with the present ideas on the synthesis of proteins, according to which protein synthesis does not occur until all the spaces in the matrix are filled with amino acids. When there is an incomplete assembling of amino acids, protein synthesis goes the more slowly, the greater the lack of amino acids.

Such a high content of free amino acids fully guarantees active biosynthesis of proteins in the seeds. The amount of protein nitrogen at the end of the ripening of the seeds reaches 98% of the total nitrogen; the content of free amino acids falls sharply. Parallel with these joint reactions of nitrogen metabolism there is complete disappearance of nicotine which shows the connection of biosynthesis and breakdown of alkaloids with nitrogen metabolism in the plant organism.

TABLE 2

Quantity of Free Amino Acids in Unripe and Ripe Tobacco Seeds (in mg % of total nitrogen)

Amino acids	Unripe (N total 3.01 %)	Ripe (N total 3.36 %)	Amino acids	Unripe (N total 3.01 %)	Ripe (N total 3.36 %)
Aspartic	130,0	2,6	Methionine	30,0	2,7
Glutamic	140,0	47,1	Leucine	120,0	8,2
Serine	50,0	2,1	Phenylalanine	60,0	4,1
Glycine	120,0	8,8	Arginine	400,0	5,3
Threonine	90,0	7,9	Histidine	290,0	20,9
Alanine	420,0	15,3	Lysine	120,0	7,6
Tyrosine	890,0	11,2	Proline	250,0	12,1
Valine	180,0	11,3	Cystine	50,0	1,9

The breakdown of nicotine is particularly sharply shown during ripening of tobacco seeds: unripe seeds contain 100 mg % of nicotine, while in ripe tobacco seeds there is no nicotine. This fact led us to study the ripe seeds of other plants of this genus of Nicotiana: N. alata, N. glutinosa, N. longiflora, N. plumbagenifolia, and N. macrophylla, in which again we found no nicotine. As to the question of the precursors of nicotine which accumulate in the seed at the beginning of its formation, whether they come from other organs of the plant or are formed at the place where the germ is formed, this still remains unsettled.

Evidently, during ripening of the seeds the activity of the enzyme systems is directed to the mobilization of all the nitrogenous substances so that they can accumulate in the reserve materials, which include the proteins. In particular, the role of enzymes in the breakdown of nicotine is shown in experiments carried out directly with nicotine preparations. Under these conditions breakdown occurs deeply enough to form ammonia, methyl amine, and simple aliphatic dicarboxylic acids [15].

SUMMARY

In the ripening of seeds, the nicotine content falls to zero.

In unripe seeds there is a high content of free amino acids. We have determined the change in content of bound amino acids and the different protein fractions of ripe tobacco seeds. We have shown an intensive accumulation of bound amino acids: alanine, arginine, lysine, and glutamic acid in ripe tobacco seeds.

These results show a direct connection between the processes of biosynthesis or breakdown of nicotine and the metabolism of nitrogenous substances.

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THE BIOSYNTHESIS OF GLUTAMIC ACID IN HOMOGENATES OF WHEAT AND PEA SEEDLINGS

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In previous work [1-4] we have shown that in homogenates of pea seedlings and in living tissue of wheat and pea seedlings there occurs an intensive process of reamination and transamination of α-ketoglutaric acid.

The present work is designed to study the synthesis of glutamic acid in homogenates from wheat and pea seedlings and to explain the changes produced in the amino acid composition of the homogenates by addition to them of the ammonium or potassium salt of α-ketoglutaric acid.

MATERIALS AND METHODS

We used 10 day old seedlings of "Moskovka" and "Ozimaya 2453" wheat, and also 10-day-old seedlings of "Rannii" peas. In order to obtain a homogeneous mass, before we prepared the homogenates we froze a definite weight of seedlings in a Dewar vessel with dry ice, and carefully ground them for 20 min in a mortar cooled with a mixture of ethyl alcohol and dry ice. As a result we obtained an entirely homogeneous fine powder, which was treated with a phosphate buffer and, if necessary, soda in the amounts indicated below. The resulting homogenate was used directly for the experiments. The experiment with the wheat and pea seedlings were carried out at pH 6.8 and 7.6-7.7. In the first case, the ground wheat powder was treated with double its weight of phosphate buffer with pH 6.8; to obtain pH 7.6-7.7 we added double the amount of phosphate buffer with pH 9.18 and anhydrous sodium bicarbonate calculated at 3 mg per g of seedlings. In the experiments with homogenates from pea seedlings, the required pH was attained by the process described in the previous work [1].

Many-times recrystallized α-ketoglutaric acid (KG), synthesized by the method of Blaise and Gault [5], was added in all cases to 0.8 ml of homogenate as a neutral solution of the ammonium (KG-NH_4) or potassium (KG-K salt to a final concentration of 0.05 M. In the experiments with homogenate from wheat seedlings the final ATP concentration was 0.01 M, and $MgSO_4$ was 0.003 M. A cozymase preparation (Co 1), obtained by the method of Williamson and Green [6], had an activity of 33% and was added as 0.2 ml of neutral solution which contained 4 mg of the preparation. Glucose was added as a powder at 10 mg per sample. The plant of the experiments with these solutions was the same as in the experiments with homogenates from pea seedlings [2]. The final volume of the experimental and control mixtures was 1.4 ml. The experimental and control samples stood for 3 hours at 37°. Reaction was stopped by addition of absolute ethyl alcohol to a volume of 10 ml. After precipitation of the protein and removal of the precipitate, 8 ml of the alcohol extract was evaporated dry in a vacuum desiccator over alkali at room temperature. The dry residue was dissolved in 1 ml of H_2O. Quantitative determination of the amino acids was carried out by the method of Kretovich and Uspenskaya [7] by chromatography on buffered paper with buffered phenol and o-cresol as solvents. It should be noted that the data obtained for alanine in experiments with homogenates from wheat seedlings can include not only alanine, but also some quantity of homoserine. Glutamine was quantitatively determined after separation from alanine in o-cresol by the method of Kretovich and Uspenskaya [8-9].

RESULTS

At first we ran experiments with homogenates from "Moskovka" wheat seedlings in which we establish the presence of reamination and transamination by addition of α-ketoglutaric acid and studied the effect on this process

TABLE 1

Effect of pH, Addition of ATP, $MgSO_4$, and Cozymase on the Synthesis of Glutamic Acid from α-Ketoglutaric Acid in Homogenates from Wheat Seedlings. Glutaminic acid in mg per 1 g dry substance; figures in parentheses: the difference, in %, with respect to the controls with water

pH of reaction mixture	ATP and $MgSO_4$			Co 1, glucose, Kg-NH_4	KG-NH_4	KG-K	H_2O
	KG-NH_4	KG-K	H_2O				
7,6	5,8 (+427)	3,8 (+245)	1,1	16,0 (+540)	10,0 (+300)	9,3 (+272)	2,5
6,8	5,4 (+1250)	1,5 (+275)	0,4	9,1 (+1200)	8,5 (+1110)	2,5 (+257)	0,7

of the pH of the reaction medium and the addition of cozymase, glucose, and also ATP and $MgSO_4$. The results are givin in Table 1.

The results in Table 1 show that at pH 7.6 there occurs the greatest absolute accumulation of glutamic acid as compared to the controls with water. At pH 7.6 in the samples with added ammonium or potassium salts of α-ketoglutaric acid there is almost the same increase in glutamic acid content. At first glance it seems that under these conditions it is difficult to distinguish reamination and transamination of α-ketoglutaric acid. However, when cozymase and glucose are added to the sample with ammonium α-ketoglutarate there is a sharp increase in content of glutamic acid, which is clear evidence of the presence of reamination of α-ketoglutaric acid by ammonium ions.

When the pH is reduced to 6.8 in the homogenate from wheat seedlings, there is a change in the ratio of reamination and transamination of α-ketoglutaric acid. Evidently the rates of the reactions of amination and transamination of α-ketoglutaric acid respond differently to a change in the pH of the reaction medium. With acidification the rate of both reactions decreases, but the rate of transamination falls to a greater degree and therefore amination of α-ketoglutaric acid becomes clearly predominant. Because of amination of α-ketoglutaric acid by ammonium ions at pH 6.8 in the homogenates from wheat seedlings 3 times more glutamic acid is formed than because of transamination of α-ketoglutaric acid with amino acids.

The addition of ATP and $MgSO_4$ at pH 7.6 to the experimental and control samples also changes the ratio of amination and transamination of α-ketoglutaric acid in the amination reactions. At both pH values the absolute amount of glutamic acid when ATP is added is considerably decreased, but the intensity of the processes which assure the synthesis of glutamic acid (excess increment of it in percent with respect to the control with water) remains at a similar level.

One of the well known inhibitors of the transamination reaction is hydroxylamine [10,11]. However, the use of hydroxylamine for this purpose is undesirable since it can react with ketoacids, forming oximes, which can be reduced in the plant to amino acids [12,13]. Also, hydroxylamine inhibits the action of the respiratory processes [14]. Therefore as a transamination inhibitor we used nicotinic acid azide (isoniazid). According to some authors [14-17], isoniazid forms a very strong complex with carbonyl groups of phosphopyridoxal and is an effective inhibitor for the transamination reaction.

In our experiments we added isoniazid to the homogenate from wheat seedlings at pH 7.6 15 min before adding ammonium or potassium α-ketoglutarate to the sample. In the experiments with high concentrations of isoniazid, we added it to the homogenate 30 min before beginning the experiment. We studied the following concentrations of isoniazid: $6 \cdot 10^{-5}$M; $2 \cdot 10^{-3}$M; $6 \cdot 10^{-3}$M; $25 \cdot 10^{-3}$M; $5 \cdot 10^{-2}$M, and $55 \cdot 10^{-3}$M. Controls were homogenates with water to which had been added the same amount of isoniazid as in the experimental samples. The results of these experiments with final isoniazid concentrations of $2 \cdot 10^{-3}$M and $6 \cdot 10^{-3}$M are givin in Table 2.

The data of Table 2 show that these concentrations of isoniazid do not decrease the accumulation of glutamic acid in samples with potassium α-ketoglutarate. We get an analogous picture using all of the above isoniazed concentration. When isoniazid is added to homogenates of wheat seedlings,

TABLE 2

Effect of Addition of Isoniazid on the Synthesis of Glutamic Acid. Glutamic acid, in mg per g dry substance; figures in parentheses are difference, in %, from the control with water

Final isoniazid concen.	Composition of sample		
	KG-NH_4	KG-K	H_2O
$2 \cdot 10^{-3}$ M	14,7 (+206)	13,5 (+180)	4,8
$6 \cdot 10^{-3}$ M	15,2 (+137)	14,6 (+127)	6,4

there is some increase in the amount of glutaminic acid in all the samples, and especially in the controls with water. Therefore the intensity of glutamic acid formation (with respect to difference between experimental and control samples) becomes less with increasing isoniazid concentration; but this occurs because of increased absolute content of glutamic acid in the control samples. In no case did we find much difference in content of glutamic acid in samples with ammonium or potassium α-ketoglutarates even when isoniazid was added in concentrations equal to or greater than the content of α-ketoglutaric acid. This could mean that isoniazid is not an inhibitor of transamination in the objects which we studied.

It is of interest to determine how a change in amino acid composition of the homogenate is produced by addition of ammonium or potassium α-ketoglutarate. The objects studied in this series of experiments were 10-day-old "Ozymaya 2453" wheat seedlings and "Rannii" pea seedlings. The data obtained in these experiments with wheat seedlings are given in Table 3.

They indicate that experiments with homogenates from seedlings of a different kind of wheat, run at a different time gave on the whole the same results as in the previous experiments. It is true that in these experiments at pH 7.6 we obtained a considerably higher increase of glutamic acid in the sample with ammonium α-ketoglutarate than with potassium α-ketoglutarate. This difference in transaminase activity is evidently due to the fact that in the experiments we used wheat of two differents kinds ("Moskovka" and "Ozymaya 2453"). These experiments showed that in wheat seedling homogenates the most active donor of the amino group in the transamination reaction with α-ketoglutaric acid is aspartic acid. This is indicated by its complete disappearance in all the samples which contain α-ketoglutaric acid.

The second active participant in the transamination reaction in the homogenate is alanine. When α-ketoglutaric acid is added at pH 7.6, its amount decreases by 88 %. Also, some amounts of glutamic and serine are used up in this reaction. Decrease in pH of the reaction mixture to 6.8 causes a sharp change in the content of some of the amino acids. For example, in samples with ammonium and potassium α-ketoglutarate we do not find any use of glutamine, and alanine is required to a much smaller extent in the transamination reaction. When ammonium α-ketoglutarate is added to the homogenate at pH 6.8 there is practically no change in serine content.

TABLE 3

Formation of Glutamic Acid in Homogenates from Wheat Seedlings. Amino acid, in mg per g of dry substance; figures in parentheses are the difference, in %, with respect to the controls with water.

Amino acids and amides	Composition, at pH 7.6			Composition at pH 6.8		
	KG-NH_4	KG-K	H_2O	KG-NH_4	KG-K	H_2O
Glutamic acid	7,15 (+361)	2,60 (+132)	[illegible] *	3,50 (+1066)	0,60 (+100)	0,30
Glutamine	5,10 (−13)	5,10 (−13)	5,90	6,75 (−1)	6,75 (−1)	6,85
Aspartic acid	0 (−100)	0 (−100)	1,85	0 (−100)	0 (−100)	0,60
Serine	1,90 (−16)	1,75 (−23)	2,30	1,30 (−41)	0,85 (−57)	1,35
γ-Aminobutyric acid	3,75 (+10)	6,25 (+83)	3,35	3,75 (−17)	6,50 (+41)	4,55
Alanine	0,30 (−88)	0,30 (−88)	2,50	1,30 (−42)	1,10 (−51)	2,25

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TABLE 4

Formation of Glutamic Acid from α-Ketoglutaric Acid in Homogenates from Pea Seedlings. Amino acids, in mg per g of dry substances; figures in parentheses are differences, in %, with respect to controls with water

Amino acids and amides	Composition at pH 7.7			Composition at pH 6.8		
	KG-NH_4	KG-K	H_2O	KG-NH_4	KG-K	H_2O
Glutamic acid	24,78 (+142)	20,09 (+87)	10,21	21,59 (+121)	14,68 (+49)	9,75
Glutamine	7,03 (—3)	6,25 (—13)	7,25	5,34 (—31)	4,37 (—44)	7,81
Aspartic acid	0 (—100)	0 (—100)	1,62	0 (—100)	0 (—100)	1,65
Serine	5,06 (—28)	5,25 (—24)	6,50	4,46 (—23)	2,18 (—62)	5,84
γ-Aminobutyric acid	7,40 (—16)	9,59 (+7)	8,81	7,50 (—6)	8,21 (+1)	8,06

All this shows the inhibition of the transamination reaction at lower pH of the reaction medium, especially in samples with ammonium α-ketoglutarate, where under these conditions the reamination of the α-ketoglutaric acid by the ammonium ion predominates. Thus, the idea which we expressed has been confirmed experimentally. In samples with potassium α-ketoglutarate we note the accumulation of γ-aminobutyric acid which forms under the action of active glutamic decarboxylase contained in the wheat seedlings [18].

In analogous experiments with homogenates from pea seedlings we obtained the same general results as in the experiments with wheat seedling homogenates (Table 4). In all cases when α-ketoglutaric acid was added to the pea seedling homogenates, aspartic acid was entirely used up. Decreasing the pH from 7.7 to 6.8 is also accompanied by a rather large accumulation of glutamic acid due to reamination of α-ketoglutaric acid by ammonium ions. However, in pea seedling homogenates acidification of the reaction medium produces a decrease in the amount of glutamine and serine which is not found in experiments with wheat seedling homogenates. Also, we do not find a difference with respect to the controls in the content of γ-aminobutyric acid in samples to which we added ammonium or potassium α-ketoglutarate. Evidently, this characteristic depends on the low glutamic decarboxylase activity of peas [19]. It is important to note that in experiments with homogenates from pea and wheat seedlings we never find synthesis of glutamine when ammonium α-ketoglutarate is added.

DISCUSSION OF RESULTS

From the results which we published earlier [1-4] and also those given in this work it follows that the path of formation of glutamic acid in plants by direct reamination of α-ketoglutaric acid by ammonia can be considered to be fully established.

The experiments with homogenates from pea seedlings and wheat seedlings show that the synthesis of glutamic acid by reamination and transamination of α-ketoglutaric acid occurs most intensively in a weakly alkaline medium at pH 7.6-7.7. Strong evidence for the presence of enzymatic amination of α-ketoglutaric acid in homogenates of these plants is the sharp increase in formation of glutamic acid in samples with ammonium α-ketoglutarate with simultaneous addition of cozymase and glucose. This shows the close connection of reamination of α-ketoglutaric acid with oxidation-reduction processes which occur in cells and the possible participation of diphosphopyridinenucleotide in this reaction as a coenzyme of glutamic dehydrogenase. In homogenates of pea and wheat seedlings there is also active transamination by the reaction of α-ketoglutaric acid with amino acids which are present in the reaction medium. The Schales showed [20] as we did that aspartic acid is the most active donor of the amino group in the homogenates, as is indicated by its complete disappearance in all the samples to which α-ketoglutaric acid was added. Other participants in the transamination reaction with α-ketoglutaric acid are alanine, serine, and in some cases, glutamine.

We have studied the effect of ATP on the biosynthesis of glutamic acid in homogenates from seedlings of peas [2] and wheat. We have shown that in pea seedling homogenates, addition of ATP to a final concentration

of 0.01 M and $MgSO_4$ to a final concentration of 0.003 M leads to an increase in content of glutamic acid in all the samples, including the controls, but in the wheat seedlings homogenates, on the contrary, the glutamic acid content falls. However, the relative rate of amination and transamination of α-ketoglutaric acid in the homogenates from both types of seedlings does not change appreciably when ATP is added. Actually, it is difficult at the present time to settle the question of the mechanism of the effect of ATP on the formation of glutamic acid in the homogenates. However, the results which we have obtained serve to show in this case ATP acts not only as a source of energy, but as a polyvalent ion which has a nonspecific action and changes the composition of the homogenates components.

In attempts to inhibit the transamination of α-ketoglutaric acid in wheat seedling homogenates by isoniazid we could never observe much difference in glutamic acid content in the samples with ammonium or potassium α-ketoglutarate, even at isoniazid concentrations which cause immediate death to animals [17], and also equal to or greater than the concentration of α-ketoglutaric acid. Evidently isoniazid is not an inhibitor of transamination in the materials which we have studied and has an entirely different action on processes connected with the biosynthesis of glutamic acid in the homogenates. It is possible that when isoniazid is added to wheat seedling homogenates there is a splitting of the azide group and the evolution of some ammonium ion which reacts with the α-ketoglutaric acid present to give glutamic acid. This idea is supported by the fact of a more appreciable increase in the amount of glutamic acid in control samples with water (to 4.8 mg and 6.4 mg) when isoniazid is added in concentrations of $2 \cdot 10^{-3}$M and $6 \cdot 10^{-3}$M compared to control samples whithout isoniazid (1.5-2.5 mg per g of dry substance).

Comparison of processes connected with the biosynthesis of glutamic acid and assimilation of ammonium ions in homogenates and live seedlings of wheat and peas [3], and also in ripening wheat ears [4] shows differences in them. Our results show that in live plants during assimilation of ammonia and its uptake into different nitrogenous compounds, a primary role is played not only by glutamic acid, but also by glutamine. This is indicated by data obtained by Kretovich, Evstigneeva, and Plyshevskaya [21] in a study of the assimilation of ammonia, labeled on the nitrogen, by various plants, and also by the results of the experiments of Kretovich and Uspenskaya [8,9] which showed that when ammonium chloride was introduced into a ripening wheat ear there was energetic synthesis of glutamine. However, we did not find this in homogenates. Evidently, the necessary conditions for the synthesis of glutamine are found in the cell structures which assure an inflow of energy from accompanying reactions required for the synthesis.

Our results show that in the live plant, glutamine is the most important source for the amino group in the transamination reaction with α-ketoglutaric acid, perhaps by direct transamination and deamination reactions combined as studied by Meister [22] and shown in plants by Olenicheva [23]. When α-ketoglutaric acid is introduced into pea and wheat seedlings, and also into ripening wheat ears, aspartic acid is not used up, and in some cases it even accumulates. In pea and wheat seedling homogenates which contain α-ketoglutaric acid, aspartic acid disappears enterily. All these facts indicate that in live plants the character of the transamination reaction with α-ketoglutaric acid is different from that in homogenates of these plants. Hence, the study of various processes in tissue homogenates serves only as evidence for the presence of a given enzyme system in them and for the possibility of the occurrence of the studied process in the live plants.

SUMMARY

In homogenates from green wheat and pea seedlings there is intensive synthesis of glutamic acid from α-ketoglutaric acid.

The optimum conditions for reactions connected with the biosynthesis of glutamic acid from α-ketoglutaric acid are concentrations of hydrogen ions corresponding to pH 7.7. Under these conditions, two reactions occur in the homogenates: 1) the transaminative reaction of α-ketoglutaric acid, chiefly with aspartic acid, and also with alanine, serine, and in some cases with glutamine; 2) reamination of α-ketoglutaric acid with ammonia.

The reamination of α-ketoglutaric acid in wheat seedling homogenates takes place with the participation of cozymase. This is indicated by the considerable increase in glutamic acid content when there is simultaneous addition of cozymase, glucose, and ammonium, α-ketoglutarate, as compared to samples in which only ammonium α-ketoglutarate was added.

Isoniazid in concentrations from $2 \cdot 10^{-3}$M to $55 \cdot 10^{-3}$M in wheat seedling homogenates does not decrease the content of glutamic acid in samples with potassium α-ketoglutarate as compared with variants which contain am-

monium α-ketoglutarate. It is possible that in this material there is no inhibition of the transamination reaction and it acts in a different way.

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THE DYNAMICS OF THE CARBOHYDRATE AND FAT CONTENT IN RIPENING AND GERMINATION OF ASH TREE SEEDS

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In ripening and germinating seeds there is intensive occurrence of breakdown and synthetic processes, in which carbohydrates take an active part. The transformations of fats and proteins are closely connected with carbohydrate metabolism. The synthesis of fats in the ripening seeds of oily plants occurs because of carbohydrates coming from other organs. In the opinion of many investigators, the use of reserve fats in germinating seeds is preceded by their change into carbohydrates [1-3]. In some cases starch accumulates with fall in the fat content [2]. In seeds which are rich in proteins, amino acids are involved in respiration, and these come from splitting of reserve proteins. Panteleev [4] considers that amino acids can either be used directly or by their preliminary transformation to carbohydrates.

Ash seeds (Fraxinus excelsior), according to our data, contain about 41% of the dry weight of "crude" protein, 25% "crude" fat, and about 20% of water-soluble carbohydrates. Almost half of the latter is the "sucrose fraction" which has a complex composition and consists of sucrose, fructosides, and easily hydrolyzed glucosides. We feel that ash seeds are an interesting material for study of the characteristics of carbohydrate metabolism in plants which contain a considerable amount of easily hydrolyzed glucosides [5].

Investigation of the dynamics of the content of sugars during photosynthesis and experiments with infiltration of monosaccharides into the leaves of this plant show a very rapid utilization of the monosaccharides without accumulation of sucrose or starch. The complex composition of the "sucrose fraction" evidently aids in rapid uptake of monosaccharides during the splitting and synthesis [5]. On the other hand, study of the respiration of leaves and buds of "glucosidal" plants gives reason to consider that compounds of a noncarbohydrate nature are then used too, probably by their preliminary transformation into carbohydrates [6]. In the present work we have studied the dynamics of the carbohydrates and fats in ripening, stratification, and germination of ash seeds.

METHODS

Collection of seeds began on August 11, in the stage of early ripeness. The seeds were freed from other fragments and fixed with steam. The mature seeds collected in September were kept until December in a dry, cold place. From December 29 to March 24 the seeds were kept in moist sand at 20° and from March 24 to June 3 in a cellar at 0-2°. Then the seeds, washed free of sand, were spread in a cuvette on moist filter paper and put in a thermostat at 20°. On June 10 samples of the sprouting seeds were taken (length of sprouts 5 mm). Then some of the cuvettes with sprouting seeds were placed in the dark and some were placed in daylight until July 2, when separate samples of rootlets and cotyledons were taken.

The seeds separated from other fragments and the sprouts were fixed by steam, dried, ground and the content in them of "crude" fat and water soluble carbohydrates was determined. The latter were separated by the method published earlier which was worked out for investigation of the carbohydrate complexes in the leaves of lilac and ash [5]. This scheme permits determination of three components of the "sucrose fraction": sucrose with the aid of invertase, and the easily hydrolyzed glucosides and fructosides by the increase in the amount of glucose and fructose after 5 min hydrolysis with 2% HCl at 70°. The sum of the carbohydrates which are relatively hard to

TABLE 1

Dynamics of Carbohydrate and Fat Content in Ripening and Stratification of Ash Seeds (in percent of dry weight)

Fraction studied	Ripening and keeping					Kept in moist conditions at temperature:			
						20°		0–2°	
	11.VIII	19.VIII	1.IX	11.IX	17.XII	29.XII	2.II	24.III	3.VI
Fructose	1,0	0,9	0,8	0,8	0,9	0,8	0,8	0,8	0,5
Glucose	0,9	0,9	0,6	0,7	0,7	0,8	0,9	0,7	0,3
Sucrose	3,0	2,6	2,8	3,2	2,6	2,2	2,0	2,1	2,2
Glucose of easily hydrolyzed glucosides	4,9	5,1	4,8	3,7	6,0	5,8	5,9	5,4	6,7
Fructosides	1,3	1,4	1,9	2,6	3,4	2,5	2,7	2,9	1,5
Glucose of difficultly hydrolyzed glucosides	6,9	7,0	7,1	9,2	7,4	7,5	7,5	5,5	5,8
Total water-soluble carbohydrates	18,0	17,9	18,0	20,2	21,0	19,6	19,8	17,4	17,0
"Crude" fats	26,9	28,6	26,4	23,1	25,0	22,0	19,0	22,0	21,0

hydrolyze and are water soluble was determined by the increase in reducing sugars after 3 hour hydrolysis with 2% HCl on the boiling-water bath. Since there is no increase in fructose during such hydrolysis and there are not even traces of starch in the ripe or germinating seeds, we call the difficultly hydrolyzing compounds glucosides. Their content is characterized as the "glucose from difficultly hydrolyzed glucosides" (as distinguished from the glucose from easily hydrolyzed glucosides). Also, we determined the invertase and amylase activity in the germinating seeds by the methods described before [7].

RESULTS

The results on the change in content of fats and carbohydrates in ripening and stratification of ash seeds is given in Table 1 which shows that during ripening and stratification of the seeds there is no especially sharp change in the ratio of fats and the various fractions of water soluble carbohydrates. During ripening there is some decrease in the amount of fats, and the content of carbohydrates rises. Just as in experiments with leaves [5,6], the most dynamic components of the "sucrose fraction" are the easily hydrolyzed glucosides, whose content falls during ripening, and fructosides, whose content increases during ripening. During ripening of the seeds after harvesting (from September 11 to December 17) the content of easily hydrolyzed glucosides and fructosides increased. There was usually no change in ratio of the most mobile monosaccharide fractions and sucrose. In this respect the results were similar to those obtained with a study of the processes of splitting and synthesis of plant "glucosides" in leaves [5,6].

Stratification also fails to produce sharp changes in ratio of the compounds studied. During the period of keeping in moist sand at 20° for 3 months the content of fats and difficultly soluble glucosides falls to a greater extent than the amount of labile components of the "sucrose fraction" and the monosaccharides. This confirms the conclusion given previously that during respiration the components of the "sucrose fraction" and especially the easily hydrolyzed glucosides play an important part in activation of the sugars and share in the mechanism of uptake of monosaccharides in one or another reaction chain to a greater extent than do the purely reserve compounds.

The results obtained by analysis of germinating seeds are given in Table 2 which shows that at the beginning of the period of germination, for 7 days, when the length of the seedling is 5 mm, there is no appreciable change in content of fat and water soluble carbohydrate. Starch is completely absent in both ripening and germinating seeds. In this respect, ash seeds differ sharply from seeds of oily plants in which even on the second to the fourth day of germination the process of transformation of fats into sugars begins energetically. For example, in flax seeds on the 12th day of germination the fat content falls by 33.5 % and the sugar content increases more than 3 times (by 9.34%). In hemp seeds the quantity of monosaccharides and sucrose increases considerably, and starch is absent in the seeds which have not germinated, but on the 10th day its content reaches 8.64% [2]. It is possible

TABLE 2

Dynamics of Carbohydrate and Fat Content in Germination of Ash Seeds. (in percent of dry weight)

Fraction studied	June 3, after stratification	In the dark				In the light	
		10.VI	2.VII	2.VII		2.VII	
		germinating seeds		root-lets	coty-ledons	root-less	coty-ledons
Fructose	0,5	0,5	0,5	0,4	0,6	0,3	0,2
Glucose	0,3	0,5	1,4	0,6	1,9	0,7	1,9
Sucrose	2,2	2,2	0,3	0,5	0,2	0,0	0,0
Glucose of easily hydrolyzed glucosides	6,7	6,4	5,5	3,5	7,0	3,6	6,9
Fructosides	1,5	1,0	1,0	0,0	1,5	0,1	0,4
Glucose of difficultly hydrolyzed glucosides	5,8	6,2	3,5	2,4	3,9	3,4	3,8
Total content of water-soluble carbohydrates	17,0	16,8	12,2	6,8	15,1	8,1	13,2
"Crude" fats	21,0	20,0	4,5	2,8	6,0	2,2	6,6

that at the beginning of germination ash seeds use nitrogenous substances whose quantity converted into protein is about 41% of the dry weight of the seeds. Evidently there then occurs a partial transformation of these compounds into sugars since it would otherwise be difficult to explain the absence of perceptible decrease in the content of monosaccharides and labile components of the "sucrose fraction". There is no reason to believe that the water soluble carbohydrates do not take part in the processes of respiration and synthesis of such compounds, for example, as cellulose. Increase in carbohydrates does not occur because of their rapid utilization. Evidently the processes of synthesis and consumption of carbohydrates occur at similar rates. Only after a month (July 2) when a considerable part of the reserve substances has been used up and the fat content fell from 21.0 to 4.5% was there found any decrease in amount of water soluble carbohydrate from 17.0 to 12.2%, with a small increase in total monosaccharides.

The separate studies of rootlets and cotyledons grown in the light and in the dark gave similar results. The sugar content in the green cotyledons was even a little lower than in cotyledons kept in the dark. This also shows the rapid utilization of the carbohydrates formed in photosynthesis. Starch was absent in all the materials studied. The absence of definite amounts of sucrose in the rootlets and cotyledons of seeds germinanting in the light may be explained by a lack of mineral compounds, especially of phosphorus. This could destroy the coordination of the processes of splitting and synthesis of sucrose. Since starch, sucrose, and other compounds can take an active part in metabolism and do not accumulate in amounts sufficient for determination, we determined the activity of amylase, invertase, and β-glucosidase in ash seeds. We could not detect amylase activity. The action of ground seedlings on a 2% starch solution for 100 hours at 36° gave no observable splitting of the starch.

The data given in Table 3 show that the invertase activity in germinating ash seeds rises and reaches the activity of this enzyme in the assimilating leaf of lilac and ash [7]. When water extracts of the germinating seeds act on a 1% solution of salicin there is a slight β-glucosidase activity (1.8 mg glucose per hour). It is possible that the low β-glucosidase activity can be explained by the unsuitability of the substrate, since it is known that the a glucone has a strong effect on the activity. The data which we have obtained indicate that in ash seeds, just as in the leaves, compounds with α-1,4-glucoside bonds do not take an active part in carbohydrate metabolism and in the processes of utilization of fats. The components of the "sucrose fraction" play the chief role, and evidently take an active part in the transformations of the different forms of glucose and fructose: α-glucopyranose, β-glucopyranose, β-fructofuranose, and β-glucofuranose, which perhaps enter into the composition of the labile glucosides.

There is every reason to believe that in ash seeds, just as in the seeds of oily plants which have been studied the utilization of fats first requires their transformation into carbohydrates, probably hexoses or hexose-6-phosphates. The resulting substances, occurring in dynamic equilibrium, are the universal reserve products and at the same time

TABLE 3

Invertase Activity in Germinating Ash Seeds (in mg of glucose per 0.25 g dry weight)

Material studied	Date of taking sample	Invertase activity
In the dark		
Germinating seeds	10.VI	4
" "	2.VII	11,2
Rootlets	2.VII	10,7
Cotyledons	2.VII	12,0
In the light		
Rootlets	2.VII	5,2
Cotyledons	2.VII	12,1

can be included in the various processes which form the components of the "sucrose fraction" whose synthesis occurs by fixing definite forms of monosaccharides. The transfer of glucosides and fructosides evidently is important in further reactions.

SUMMARY

In ripening and stratification of ash seeds there is no great change in the ratio of water soluble carbohydrates and fats. Among the components of the "sucrose fraction" the labile glucosides and fructosides are relatively more dynamic. There is almost no sucrose in these processes.

In the first phase of germination of ash seeds there is no great change in the ratio of the different fractions of carbohydrates and fats, and only later does the content of the latter fall several-fold with some decrease in amount of the "sucrose fraction" and difficultly hydrolyzed glucosides, and an increase in total monosaccharides. The resulting data give a basis for belief that in germinating ash seed the utilization of fats is preceded by their transformation into carbohydrates. The latter do not accumulate because of their rapid use which favors the complex composition of the "sucrose fraction".

Neither starch nor active amylase is found in resting or germination ash seeds. Evidently, compounds with α-1,4-glucoside bonds do not take part in carbohydrate metabolism in these seeds.

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THE METHOD OF PAIRED STEREOIMMUNOLOGICAL DEVELOPMENT OF BLOOD SERUM PROTEIN ELECTROPHORETOGRAMS

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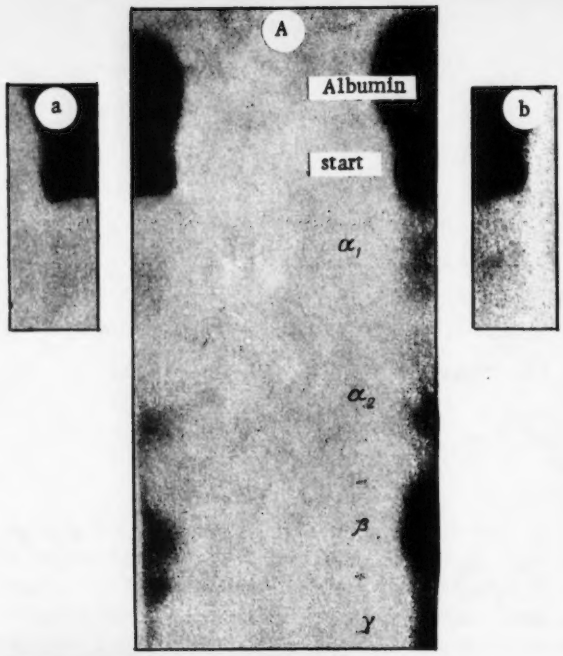
The method which we describe for immunoprecipitation analysis of proteins in agar gels has a number of precursors. The most recent of these is the method of Oudin [1] in which a mixture of protein antigens diffusing through a layer of agar meets a front of diffusing antibody; thereupon the immunoprecipitates which correspond to each antigen-antibody pair appear in the form of a line. A further development of the immunoprecipitation method is the procedure of Ouchterlony [2], used for the comparison of two antigens under investigation. Comparison is carried out on the basis of a principle which can be called paired immunological development.

Each of two sets of antigens reacts with the developing antiserum not separately, but in one combined system. Two sets of antigens and antisera diffuse toward each other from three points in the agar gel, so that in this diffusing system a line is not formed, but a solid cylinder. When two identical antigens are present in the comparison mixture, they form one continuous precipitation line. With entirely different antigens in the agar, they form two separate planes. Two proteins which have the same antigen group, but also contain different antigenic groupings give the so-called "spur" phenomenon (reaction of "incomplete identity"). The method of Ouchterlony is particularly valuable for showing this latter very fine distinction between two antigens, which is not shown by the methods of Oudin or of Grabar [3]. The latter is also an immunoprecipitation method for analysis of protein mixtures which have first been submitted to electrophoretic fractionation. If we make a comparison of two proteins mixtures by the method of Grabar, we are able to detect: 1) the disappearance of one of the components of the mixture (or the appearance of a new component); 2) a change in the electrophoretic mobility of the protein even if it has not changed with respect to its antigenic power, that is, it has retained all its antigen groups; 3) a change in the molecular weight, since this can be indicated by the distribution of the corresponding precipitation lines.

In the present work we have assumed a new principle of immunological development which permits comparison: in a paired reaction of an antigen at any point on the electrophoretogram with an antibody of the same fraction (homogeneous as to mobility) by a second study of the electrophoretogram. Thus, in our method, as distinguished from the method of Grabar, we have used the principle of paired immunological precipitation analysis of Ouchterlony, in which the triad antigen I - antigen II - antiserum takes part in the reaction of mutual combination. At first, for the paired development of two electrophoretograms, we cut out the corresponding fractions from electrophoretograms run parallel with each other, for example, the albumin fraction of two sera, and when these pieces had been put into warm, setting agar, we carried out the Ouchterlony reaction.

In Fig. 1 we give a photograph of the preparation obtained by this method. For setting up the Ouchterlony reaction we used in this case pieces cut from the albumin portion of two electrophoretograms of the blood serum of two healthy rats. We see in the preparation two pairs of immunoprecipitation lines, fused two by two because of identity of the given antigen proteins. We then replaced this primitive and difficult operation by the method of stereodevelopment which consists in the following.

The electrophoresis was carried out in a special bridge-shaped cuvette with side rims. The starting cells in the agar, filled with a mixture of the studied serum and agar, reached to 6 mm from the bottom of the cuvette (Fig. 3). Before we poured the agar, we placed on the bottom of the cuvette, parallel to the axis of electrophoresis, a piece of wood with a semicircular cross section of 3 mm and a length of 45 mm as a form for the gutter, which was to be filled with antiserum. After running the electrophoresis we removed all the agar mold with the electro-



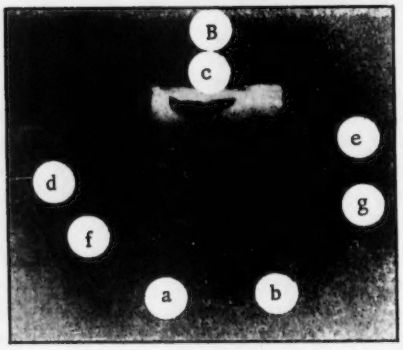


Fig. 1

Fig. 2

Fig. 1. Electrophoretograms (A) and Ouchterlony reaction (B) of blood serum of two healthy rats. Reaction carried out with pieces a and b cut from the albumin section of the electrophoretogram. α, β, γ) corresponding globulin fractions ; c) cell with antiserum; d,e and f, g) two pairs of precipitation lines. The one dimensional lines formed by identical antigens are fused.

Fig. 2. Cross section of a stereoimmunological electrophoretogram. The section is cut through the α_1-globulin portion of the stereoimmunological electrophoretogram of mouse and rat blood serum. a) "Spur" ; b) immunoprecipitate dome corresponding to mouse antigen; c) immunoprecipitate dome corresponding to rat antigen; d) precipitate dome of rat antigen which has no immunological relation in common with mouse antigen; M and R) positions of electrophoretograms of mouse and rat blood sera; G) position of the gutter with the antiserum.

phoretogram in it from from the cuvette, and, turning the bottom up, we filled the gutter with antiserum. Immunological development lasted 4 days at 4° . With this relationship of the electrophoretogram and the gutter with the antiserum there was formed a spatial, three-dimensional immunological surface, in most cases with a dome shape. The domes penetrated each other, fusing or forming one inside the other (Fig. 4). In sections running

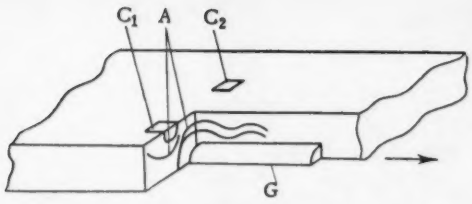


Fig. 3. Technique of running stereoimmunological electrophoresis. C_1, C_2) Starting cells, filled with a mixture of the serum being studied and agar; G) form of the gutter for the antiserum; a) precipitation surfaces in the cross section. Arrow indicates direction of electrophoresis.

across the axis of electrophoresis, it is possible to see a picture which resembles the immunograms of the Ouchterlony method, and on longitudinal sections, a resemblance to the immunoelectrophoretograms of Grabar.

The exact location of the cross section of a definite serum protein fraction is determined with the help of a control electrophoretogram run in parallel and stained without preliminary immunological development. In Fig. 2 we give a photograph of one of the cross sections of a paired stereoimmunoelectrophoretogram of rat and mouse serum, developed with antirat (rabbit) antiserum. In the section, cut through the α-globulin fraction, we see the typical reaction of incomplete identity. Our method thus permits us to show incomplete identity of antigens with the same mobility on two different electrophoretograms, which is impossible to show by other methods. We use this method in all cases when we are seeking new, unknown antigens.

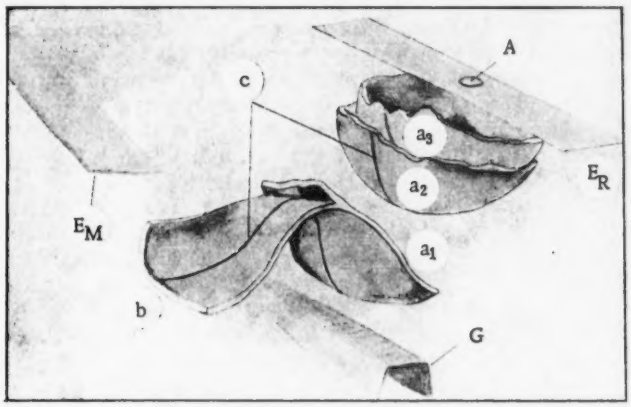


Fig. 4. Plan of precipitation surfaces. a_1, a_2 and a_3) Precipitation surfaces formed by antigens of spot A of the rat serum electrophoretograms (E); b) surface formed by antigen of mouse serum; E_M) electrophoretogram of mouse serum; G) gutter from which antiserum diffused; c) line formed by the plane of the vertical cross section. Surfaces a_1 and b form a figure which gives in cross section the "spur" phenomenon.

SUMMARY

We describe a new method which is a combination of the electrophoretic separation of proteins and the immunological development using the principle of the paired reaction of Ouchterlony. The immunological development process is three dimensional and the immunoprecipitation surfaces form in the shape of domes. The method permits us to show incomplete identity of two antigens with simultaneous determination of their electrophoretic mobility.

The method is recommended for study of very complex mixtures of antigens (for example, blood serum) which are difficult to investigate by the method of Ouchterlony.

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THE PREPARATION AND PROPERTIES OF THE I^{131}-ANTIGEN OF DYSENTERY BACTERIA

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The study of the dynamics of assimilation, distribution in the organism, and removal of bacterial antigens, like the study of the reaction of antigens and antibodies in the living organism and in experiments in vitro, has great interest for immunologists and immunochemists. There is special significance in connection with the possibility of obtaining and using isotopically labeled microbe antigens with a high specific activity; these would permit the injection into animals of small amounts of the bacterial antigens, which as a rule are toxic. However, it is important that the labeled group or atom which is introduced should not change the specific antigenic properties of the antigen.

The use of I^{131} for labeling bacterial antigens has a number of advantages, since the iodination reaction, well studied for proteins [1-6], is simple to carry out, requires no special apparatus, and does not change the specific antigenic properties of the proteins during combination of indicator amounts of iodine. It can be assumed that an analogous picture will be found in the iodination of microbial antigens which contain proteins in their composition.

Since most microbial antigens are firm complexes of proteins, lipids, and polysaccharides in different combinations, the possibility of iodination of these complexes should depend on the relative size of the protein part of the antigen molecule. A considerable role is also played by the presence of tyrosine in the molecule, since iodine under the reaction conditions described below combines chiefly with the phenol ring of tyrosine. Similar reactions are possible with histidine and cysteine, but they occur to a slight extent only and are usually not even considered. In the case of iodination of an antigen which contains lipids with unsaturated linkages it is important to consider the possibility of addition of iodine to this part of the molecule, with rupture of the double bonds. The possibility of formation of a bond between iodine and polysaccharides of some types is also not excluded [7].

We were interested in the use of I^{131} for introduction into bacterial antigens from the viewpoint of the possibility of this reaction and also of the strength of the bond between the antigen molecule and the iodine after injection into the animal organism. The strength of the chemical bond is not in doubt here, since iodine is not split from tyrosine even by strong acid or alkaline hydrolysis of the protein. However, it is very important to consider the possibility of enzymatic splitting of the compounds injected into the organism.

We know of only one study, by Seligman et al. [8], on the iodination of a specific antigen of the polysaccharide type from Serratia marcescens (Bact. prodigiosum) and some studies from the same laboratory which describe the use of this preparation in experiments on animals [9, 10]. The authors of a second study [11] also using iodinated bacterial antigen (the somatic antigen of Flexner bacteria, type III Z) used chiefly another method the reaction of antigen coupled with diazotized I^{131}-aniline. This method, which changes the specificity of the antigen and is more complicated technically, gave a slightly active preparation. The work of Masouredis [12] concerned iodination of diphtheria toxin of a protein character and did not differ from the ordinary work with iodinated proteins.

EXPERIMENTAL PART

Obtaining the Antigen and Its Iodination

The antigen of Flexner dysentery bacteria (strain 26) was obtained from an acetic acid extract of dried bacteria (0.1 N CH_3COOH, from one to one and a half hours on the boiling water bath) by precipitation with 6 volumes of ethanol without preliminary evaporation of the extract (modified method of White [13]). Before precipitation by alcohol the extract was dialyzed for 2-3 days against running water and centrifuged to remove the small amount of flocculent precipitate which formed on the walls of the cellophane bag, apparantly a protein derivative. The precipitate which formed in the refrigerator in the course of a day was centrifuged, washed twice with alcohol and once with ether, and dired in a desiccator.

Determination of total nitrogen in the precipitate by the micro-Kjeldahl method gave values from 2.5 to 3.2 % for preparations obtained at different times. The content of total phosphorus was 1.5-2.5%. The preparation had clear antigenic and serological properties. Whole serum obtained from rabbits immunized by the preparation (immunization 6 times with two-day intervals, increasing the amount of antigen from 0.1 to 1.0 mg) gave a ring of precipitation with the antigen in dilutions of 1: 100,000 to 1:1,000,000.

The antigen precipitate gave a good reaction for polysaccharides by the Schiff reaction on paper, and dissolved well in water and physiological saline at concentrations of 1-5 mg/ml. The yellowish, slightly opalescent solution of antigen did not give a precipitate with TCA and the usual protein precipitants, but the chromatographic method showed the presence in a hydrolyzate of the preparation of a whole series of amino acids. Acid hydrolysis of the antigen was carried out especially to show the presence of tyrosine in the complex, and it was found in amounts less than 0.5%. Tyrosine was determined by the diazo reaction on paper (modification of the method of Pauli) and by the reaction of coloring with ninhydrin. It is possible that the values given for the tyrosine content are somewhat low, since the possibility of partial splitting of tyrosine during acid hydrolysis in the presence of monosaccharides is not excluded; such monosaccharides would be split from the polysaccharide part of the molecule [14]. The reaction of precipitation in a gel by the method of Ouchterlony [15] with a specific antiserum gives one band of antigen precipitation.

For iodination of the protein we used the method of Blum and Straus [16] in the modification of Wormall [17] worked out by Banks [18]. The reaction was carried out on a water bath at 37-45° with constant stirring by an electromagnetic stirrer. The iodination mixture was prepared 15-20 min before beginning the reaction; it consisted of the following components: NaI^{131}, active solution without a carrier (2-10 ml); KIO_3, 1 drop of saturated solution; NaI, carrier, (0.05-1% solution), varying in amount from 0.025 ml to 0.1 ml; 2 N H_2SO_4, 2 drops.

The antigen solution was made alkaline to pH 8-9 with a solution of ammonia. The iodination mixture was added dropwise to the antigen over 30-40 min. Since there was a possibility of shift in the pH, it was controlled periodically and compensated for by adding ammonia solution. The reaction mixture remained at room temperature for one hour, then was dialyzed for two days against running water.

The activity of the antigen precipitated before dialysis fell more than double after 2-hour dialysis and then changed very little even after 6-day dialysis. The activity of the antigen did not change appreciably by repeated reprecipitations with alcohol. The antigen complex was precipitated from the dialyzed solution with alcohol, allowed to stand in the refrigerator overnight, centrifuged, washed twice with alcohol and once with ether, and dried. The serological and antigenic properties of the iodinated antigen were not changed to any extent. The latter fact corresponded to the literature data concerning proteins of antigens. For a test we immunized a rabbit with unlabeled and iodinated antigen and set up a cross reaction of precipitation with both antisera and antigens; the experiments showed the identity of labeled and unlabeled antigen preparations.

After working out this method of iodination for use with our antigen, we tried to establish the relation of specific activity of the resulting substance to the specific activity of the original I^{131} preparation, to the amount of carrier NaI added to the iodinating mixture, to the initial concentration and amount of antigen in the solution, and, finally, to the total amount of added activity. In Table 1 we give the results of a number of experiments on iodination of the antigen preparations.

The activity of the antigen complex was expressed in μC per mg with consideration of the effectiveness of the count (equal to 7%) of the counter which we used. The degree of uptake of activity of I^{131} by the antigen

TABLE 1

Relation of Activity of I^{131}-Antigen to Activity of I^{131} Used in the Reaction and to the Amount of Carrier NaI

Nature of Flexner 26 of antigen ser. & total N, %	Total activ. used in reaction, mC	Amount of carrier NaI	Activ. of iodinated antigen		% of binding activ. per wt. of antigen
			counts per min · mg	μ C/mg	
№ 752	0,7*	0,1 ml, 0,5%	50 400	0,32	6,6
Ntotal=2,88	1,4*	0,1 ml, 0,5%	182 000	1,17	12,0
	2,1*	0,1 ml, 0,5%	268 000	1,73	11,8
№ 752	0,7*	0,1 ml, 0,05%	66 500	0,42	14,3
Ntotal=2,88	2,1*	0,1 ml, 0,05%	190 000	1,21	13,3
№ 300	0,7*	0,1 ml, 0,5%	65 000	0,42	11,5
Ntotal=2,62	0,7**	0,1 ml, 0,5%	75 000	0,47	13,3
	0,7***	0,1 ml, 0,5%	70 000	0,45	13,0
№ 410	10,0*	0,1 ml, 0,5%	435 000	2,80	5,1
Ntotal=2,66	10,0*	0.05 ml, 0.125%	434 000	2,80	5,0
№ 300	2,1*	0,1 ml, 0,5%	120 700	0,79	5,0
Ntotal=2,94	4,2*	0,1 ml, 0,5%	308 000	2,00	6,5
	6,3*	0,1 ml, 0,5%	631 300	4,00	9,2

100 mg antigen used, concentration: * 10 mg/ml; ** 2 mg/ml; *** 50 mg/ml .

was calculated starting from the rating data with correction for decay during the period of the experiment. The relation of the final activity of the antigen to the amount of carrier NaI could not be established , as is natural since in the reaction we used an amount of carrier known to be less than that needed for full saturation of all the tyrosine residues of the antigen. The amount of binding of iodine also does not depend on concentration of antigen in the solution (marked by asterisks) or on the specific activity of the original I^{131} solution. In the iodination of one antigen preparation with increasing amounts of I^{131} activity and the same quantity of carrier NaI we could also find increasing final activity of the antigen.

As Table 1 shows, and also from many experiments not shown in it, iodination of the antigen occurs, and the activity of the preparation is sufficiently high because of the use of a minmum amount of carrier NaI and a high specific activity of the iodinating mixture. The degree of uptake of I^{131} by the antigen in most cases is between 10 to 15% and in some cases reaches 20%. A number of experiments, however, showed only half as great an uptake. The degree of uptake of I^{131} is somewhat lower than that given in the literature for proteins.

Iodine is combined quite strongly with the antigen and is not removed by long dialysis and reprecipitation. Nonspecific precipitation of the antigen from water solution by alcohol results in precipitation of 85-95% of the activity, and the activity which remains in the supernatant liquid is also due to antigen partly solution in alcohol.

Determination of the Purity of the Iodination Preparation

It seemed to us that the most suitable test for determining the purity of the I^{131}-antigen was the reaction of precipitation with a specific antiserum.

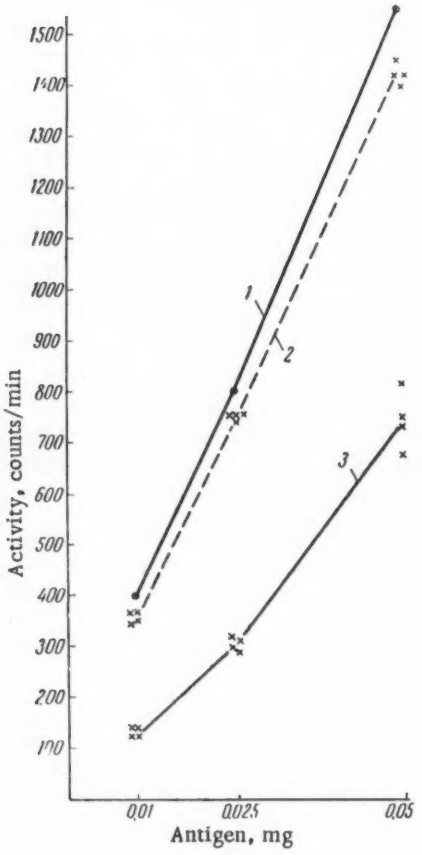
For comparison the precipitation reaction was also run with I^{131}-globulin and P^{32}-antigen. The latter was obtained from Flexner dysentery microbes (strain 26) grown on a medium which contained $Na_2HP^{32}O_4$ and was purified by dialysis and reprecipitation. The activity of the P^{32}-antigen varied between 0.2 and 0.3 μC per mg (40,000-100,000 counts/min·mg), that is , it was about on the order of the least activity of the I-antigen.

The total globulin fraction of normal horse plasma , obtained by salting out with a half-saturated ammonium sulfate solution, was iodinated by the method described above. After iodination of the dialyzed fraction it was repeatedly reprecipitated (up to 5 times) and washed with half-saturated $(NH_4)_2SO_4$ solution. A second dialysis, like the first , was carried out at 4° against repeatedly changed 0.9% salt solution to disappearance of traces of ammonia (test with Nessler reagent). The amount of protein before and after iodination was determined by total nitrogen with the use of the coefficient 6.25 . The serological properties of the iodinated protein were not changed.

TABLE 2

Precipitation of Various Preparations of I^{131}-Antigen by Specific Antisera (amount of antigen, 0.1 mg/ml)

Amount of antiserum, ml	Activ. of precipitate, counts/min	Activ. of supernatant, counts/min	Σ activ. of precipitate and supernatant	Theoret. activ. of precipitate at 100% precipitation, counts/min	Activ. ratio of precipitate and supernatant
0,5	1660	2010	3670	3620	1:1,1
	1715	1815	3530		1:1,0
1,5	1640	2080	3720		1:1,1
	1555	2040	3595		1:1,3
0,5	5160	5640	10800	9240	1:1,0
	5255	5940	11195		
1,5	5550	6160	11710		1:1,0
	5725	5840	11565		1:1,0
0,5	9845	10665	20510	18940	1:1,1
	10290	11250	21540		1:1,1
1,5	11540	10560	22100		1,1:1,0
	11485	11280	22765		1:1,0



Precipitation of P^{32}-antigen by specific antiserum: 1) theoretical curve for 100% precipitation; 2) sum of activity of precipitate and centrifugate; 3) activity of precipitate. In the sample we used 0.5-0.25 ml of antiserum.

Rabbits were immunized to the globulin fraction (9 injections over 2 days, from 1 to 3 mg of protein). The antiserum was used in experiments on the precipitation of the iodinated globulin fraction. All the activity of the iodinated protein (more than 99%) was precipitated by TCA (final concentration 3-5%).

The experiments were as follows: various amounts of antigen and the corresponding antiserum were mixed in centrifuge tubes; the mixture was kept in the thermostat at 37° for 3-4 hours. After formation of a precipitate in a refrigerator (1-2 days) the supernatant liquid was centrifuged off and the activity of the centrifugate was determined. At this stage, according to the data of Tolmage and Maurer [19] and Banks et al. [18], and also in the following steps of washing the precipitate it is important to use a cooled centrifuge. The authors emphasize the possibility of partial solution of the resulting antigen-antibody complex. We carried out centrifuging without cooling. The precipitate was washed twice with cold 0.9% NaCl solution, dissolved in a small amount of 0.1 N NaOH (0.5-1.0 ml), and then its radioactivity was determined (0.1-0.2 ml, three parallel tests). It is important to note that we carried out precipitation of the antigen in a zone of excess antibody which, according to present ideas, leads to full combination of the antigen in a complex with antibody.

We also carried out digestion of labeled antigen by a commercial preparation of pepsin according to the generally used method described for bacterial antigens by Zaitseva [20]. After digestion, the enzymatic hydrolyzate was dialyzed for two days against running water and centrifuged. The residual antigen after digestion was precipitated and dried as usual by alcohol and ether. We determined the activity of the resulting precipitated and the total nitrogen which it contained.

With none of the experiments and with no antigen or globulin preparation labeled with I^{131} or P^{32} did we obtain

TABLE 3

Precipitation of the Digestion Product of I^{131}-Antigen with Antiserum for the I^{131}-Antigen

Antigen preparation	Amount of antiserum, ml	Activ. of initial I^{131}-antigen, counts/min	Activ. of precipitate, counts/min	Activ. of supernatant, counts/min	Activity of precipitate / Activity of supernatant
Product of digestion of I^{131}-antigen, 0.01 mg	0,5	1075	75	720	1:10
	0,5		340	750	1:2
	1,0		655	473	1,5:1
	1,0		655	550	1:1
	2,0		395	861	1:2
	2,0		535	693	1:1,5
Initial I^{131}-antigen before digestion, 0.01 mg	0,5	5,300	2340	5290	1:2
	0,5		2020	2900	1:1
	1,0		2545	2673	1:1
	1,0		1755	2827	1:1,5
	2,0		930	3549	1:4
	2,0		1065	3612	1:3

complete precipitation of the activity or even close to 100% of it. Under different experimental conditions the ratio $K = \frac{\text{activity of precipitate}}{\text{activity of supernatant liquid}}$ in most cases was 1, and sometimes it varied because of change in activity of the precipitate.

The ratio K remained unchanged during precipitation of the same amount of antigen with three or more times the amount of antiserum, which excludes the possibility of insufficient antigen for precipitation. Also, the supplementary reaction of ring precipitation with the supernatant liquid after separation of the main mass of precipitate is negative, that is, unprecipitated antigen is not present.

In Table 2 we give the results of three characteristic experiments with different preparations of I^{131}-antigens.

The results in Table 2 show that the activity of the I^{131}-antigen is distributed equally between the activity of the specific precipitate and the activity of the centrifugate, and this ratio does not change either when the amount of antiserum or of antigen is increased. In the figure we show the results of precipitation of P^{32}-antigen by specific antiserum, combining the values obtained by precipitating the antigen with 0.5 ml and 0.25 ml of antiserum; the results do not differ from those given in Table 2 for the I^{131}-antigen.

Analogous results were obtained in the precipitation of iodinated total globulin fraction of horse serum by specific rabbit antiserum.

Thus, the results of the experiments on precipitation of antigen show that specific antisera under the conditions of our experiments precipitate about half the activity of I^{131}-antigen, I^{131}-globulin, and P^{32}-antigen. At the same time, precipitation of these preparations by a nonspecific precipitant gives 99% precipitation of all the activity in the case of I^{131}-globulin and 85-95% in the case of I^{131}-antigen.

Since labeled antigen was supposed to be used in experiments in vivo after per os introduction into animals it was of interest to see whether the iodine-containing protein part of the antigen molecule was split by proteolytic enzymes. At the same time we wished to see whether the distribution of the activity of the precipitate and supernatant liquid was changed after precipitation by specific antisera of the heptene formed as a result of digestion of the antigen and to a considerable extent freed from possible polypeptide contaminants.

As a result of three experiments on pepsin digestion in vitro of three preparations of I^{131}-antigen, we showed that the total nitrogen content in the product of digestion (antigen after enzymatic hydrolysis) decreased more than double and the activity decreased four-to fivefold. Hence, along with the loss at the time of dialysis, the loss in digestion gives a disappearance of a large part of the activity of the antigen.

Experiments with precipitation of precipitates obtained after digestion of the original I^{131}-antigen by antiserum showed that the digested preparation did not lose its serological properties and was precipitated by the antiserum with the usual distribution of activity between the precipitate and the supernatant liquid. In Table 3 we

give results of one of these experiments. The table shows that the character of the distribution of activity between the specific precipitate and the supernatant liquid is not changed after further purification of I^{131}-antigen by digestion and dialysis.

DISCUSSION OF RESULTS

Our experiments have shown that the glucoprotein fraction isolated from Flexner dysentery bacteria can be iodinated to give a sufficiently highly active preparation without changing its antigenic and serologic properties. On the basis of the experiments on digesting the antigen we can assume that the chief part of the activity is combined in the ordinary way with the tyrosine residues of the protein part of the glucoprotein. The rest of the activity can be adsorbed by the polysaccharide part of the antigen or combined with lipid components which may be present.

The best results in the iodination reaction are usually obtained at antigen concentrations of 10 mg/ml and a total amount of it which does not exceed 100 mg. Activity of the antigen needed for work on large laboratory animals (rabbits, dogs) was obtained by using an activity in the initial iodine solution of 2 to 5 mC per 100 mg of antigen. For work on small animals sufficient antigen activity is obtained by using an activity of I^{131} not exceeding 1 mC.

The results in the experiments on precipitation of the antigen with antiserum can be treated differently. Unprecipitated activity of the microbe antigen could be considered related to contaminants in the solution if an analogous picture were not obtained with iodinated plasma globulin, each of the fractions of which has antigenic properties and should therefore be precipitated by antiserum. Explanation in terms of impurities of a protein nature becomes still more doubtful if we remember the results on precipitation of I^{131}-antigen after its digestion by pepsin followed by dialysis and reprecipitation of the products of digestion. In these experiments it was shown that the distribution of activity in these products after precipitation with antiserum was enterely analogous to the distribution of activity of all the preparations which were used, even though a considerable part of the nitrogen-containing compounds were removed after digestion and dialysis. Errors of method may have existed, but it is difficult to suppose such a large solubility of the antigen-antibody complex at room temperature.

A number of authors have shown the occurrence in antiserum of unsaturated antibodies which can give unprecipitated complexes.

In the reports of the International Conference on Peaceful Uses of Atomic Energy in 1955, in the paper of Dickson [21] there is a direct reference to the existence of unsaturated antibodies at the point of equivalence in ordinary antisera, and the necessity to consider this in designing experiments on the precipitation reaction. In the communication of Feinberg [22] on working out a method for determining unsaturated antibodies, and of Becker and Feinberg [23] there are data analogous to ours. Sternberger, Feinberg, and Clarke [24] made a study of unsaturated antibodies and worked out a method for their isolation. In the work of Masouredis [12] it was shown that precipitation of the I-toxin of diphtheria bacteria in the zone of excess antibody took place up to 80%.

In this work we cannot analyze this question deeply, but this will be the aim of our next investigation.

SUMMARY

We have obtained an iodinated bacterial antigen of a polysaccharide nature with a sufficiently high specific activity (1-5 μ C per mg) and without change in serological and antigenic properties.

In the precipitation of the io iodinated antigen by specific antiserum, about 50% of the antigen activity passes into the precipitate.

The product obtained after digestion of the antigen complex by pepsin and subsequent dialysis loses 75-80% of the initial activity. The content of total nitrogen in the preparation falls to about half.

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THE IN VIVO AND IN VITRO METABOLISM OF CARBAMYLASPARTATE IN ANIMAL TISSUES

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The knowledge of pyrimidine nucleotide biosynthesis is scarce compared to that relating to the corresponding purines. It is known, at least in the case of normal rats, that the free pyrimidine bases are ineffective as precursors in nucleic acid synthesis [1-3]. Orotic acid (uracil-4-carboxylic acid) is known to be a common precursor of all nucleic acid pyrimidines [4-6]. In investigations of orotic acid biosynthesis carbamylaspartate (CA) was shown to be an apparantly direct precursor of the former compound; it was shown to be synthesized in the liver of rats [7]. A study of the general metabolism of pyrimidines in the organism is thus possible through following the fate of CA. The present work deals with such a study, using CA labeled with C^{14}, on batches of normal mice and rats, and of those bearing various transplanted tumors.

METHODS

CA, labeled with C^{14} in the ureidocarbon, was prepared from aspartate and $KC^{14}N$, by the method of Nyc and Mitchell [8]. The synthesis was accomplished in two stages. The first step involved the oxidation of $KC^{14}N$ to $KC^{14}NO$; in the second step the $KC^{14}NO$ was allowed to react with aspartic acid in acid solution, when C^{14}-carbamylaspartate precipitated. The product was twice recrystallized from hot water.

Analytical data for the product were as follows:
Theory: M.p. 178-180°; C – 34.10; H – 4.56; N – 15.91
Found: 177-178° ; C – 34.10; H – 4.74 ; N – 15.95 .

The product was checked chromatographically for radiochemical purity, and on the developed paper chromatograms only one radioactive peak, corresponding in position to CA, was found. The specific radioactivity of the synthesized CA was 1.9 mC.

The animals used in the experiments were mice bearing Ehrlich ascitic carcinoma and also C_3H strain mice with transplanted hepatoma (strain developed by Gel'shtein [9]); normal rats; rats bearing sarcoma-45 and ascitic hepatoma (Zazhdel' strain). Labeled CA in neutral solution was introduced subcutaneously, intraperitoneally, or directly into the tumor, in amounts ranging from 7,000 to 8,000 cmp/g weight. The animals were killed at various specified intervals after label administration. The tissues were processed by a slightly modified procedure of Schmidt and Thannhauser [10]. The tissues were homogenized with five volumes of cold, 5% TCA, or 0.2 N $HClO_4$. Dry, fat-free powders of the tissues, containing the nucleic acids, were obtained by extraction of the acid-insoluble pellet with a mixture (3:1) of ethanol and ether at 80° for 3 min. Each sample was extracted twice. The residue was dried with ethanol and ether. In order to isolate the nucleic acids the powders were suspended in 0.3 N KOH and allowed to hydrolyze at 37° for 18 hours. The hydrolyzate was neutralized and centrifuged; the supernatant contained the RNA, while the DNA remained in the precipitate. Acid-soluble compounds of the tissues were available from the perchloric acid extracts, which were neutralized with KOH and cooled; after removal of the precipitated $KClO_4$ samples of these solutions were taken for the estimation of radioactivity. This fraction will be referred to as the "acid soluble compounds". Nucleic acids were also extracted from the acid-

TABLE 1

The incorporation of CA into the Tissues of Normal Mice (in cpm/10 mg)

Tissue	Incorporation time, hours	
	3	24
Kidneys	102	65
Liver	20	43
Intestinal mucosa	13	24
Spleen	10	3
Testes	7	7
Brain	5	6
Thymus	10	7

TABLE 2

The Incorporation of CA into the Nuclear and Mitochondrial Fractions and into the Supernatant of Normal Rat Livers and of Rat Sarcoma-45 (in cpm/10 mg)

Tissue	Nuclei	Mitochondria	Supernatant fraction
Liver	88	62	13
Sarcoma-45	15	8	6

insoluble material by heating at 90° for 15 min in 0.2 N $HClO_4$. The extraction procedure was repeated once. The extracts were pooled, neutralized with KOH and, after removal of the $KClO_4$, samples were taken for estimation of radioactivity.

Radioactivity excreted in the urine was estimated directly on various samples of urine dried directly on the planchets; the results were corrected for self-absorption. Radioactivity was estimated with an end-window counter MST-17, in an apparatus of the B-2 type. Data were expressed as cpm/10 mg of sample, or as the relative specific activity (in cpm/10 mg sample x 100, over activity introduced per g weight).

RESULTS

A study of radioactivity excretion showed that over 50% of the introduced label was excreted in the first 2 hours after administration; further excretion through the urine was insignificant. According to Anderson et al. [11]. Very little CA is excreted in the feces, while its elimination as expired CO_2 comprises less than 5% of the administered dose in the first 2 hours and reaches 20% in 24 hours.

The Incorporation of CA into Various Tissues, Subcellular Fractions and Nucleic Acid Fractions

The incorporation of CA into the nucleoproteins of various tissues in normal mice were studied over the 3- and 24- hour period. The results are presented in Table 1.

It will be seen from the table that the incorporation of CA into the nucleoproteins of brain, thymus and testes was very small. There was considerable incorporation of label in to the kidneys and liver nucleoproteins, and it would appear that the kidneys serve mainly as eliminating system for CA, since the activity there decreased considerably at 24 hours, while in the liver, and also in the mucosa and spleen, the radioactivity accumulated.

In order to determine the intracellular distribution of the CA normal rat livers and sarcoma-45 tissue were fractionated. The tissues were homogenized in 10% sucrose to give a 10% suspension. The nuclear fraction was sedimented at 600 rpm. The supernate was centrifuged at 8,500 rpm to sediment the mitochondria. The fraction remaining after the sedimentation of the mitochondria was termed the "supernatant fraction".

The results dealing with the incorporation of CA into the subcellular fraction of normal rat liver and rat sarcoma-45 are presented in Table 2, from which it is seen that CA was incorporated most intensively into the nuclear and mitochondrial fraction. The high degree of CA incorporation into nuclei was also mentioned by Reichard [12]. Hurlbert and Potter [13] reported a very high activity of the nuclear fraction in the incorporation of C^{14}-orotate.

It was of interest to determine the distribution of the CA label within the tissue nucleoproteins. In no case was any label found in the proteins of the tissues investigated. The distribution of the labeled CA in the tissue nucleoproteins of normal rat liver is shown below:

Nucleoproteins	RNA	DNA
484	421	15
328	312	10
286	285	5

Thus, practically all the label was found in the RNA; the activity in the DNA was insignificant. This distribution held for all the periods after administration employed, regardless of the amount of label given or the route of administration.

TABLE 3

The Incorporation of CA into the Tissues of Normal and of Tumor-Bearing Rats (relative specific activities)

Tissues	Acid-soluble compounds. Hours after CA administration						Nucleic acids. Hours after CA administration					
	2		24		48		2		24		48	
	I	II	I	II	I	II	I	II	I	II	I	II
Liver	3,95	5,25	0,32	0,60	0,25	0,13	0,99	4,05	0,88	1,87	1,79	1,55
Kidneys	20,81	28,30	0,35	0,43	0,23	0,17	3,54	4,59	1,23	1,49	1,08	0,79

TABLE 4

CA Incorporation into Various Tumors (relative specific activities)

Type of tumor	Acid-soluble compounds; hours after CA administration		Nucleic acids; hours after CA administration	
	2	24	2	24
Sarcoma-45	0,31	0,05	0,12	0,08
Ascitic rat hepatoma	0,11	0	0,05	0
Ehrlich carcinoma	0,60	0,12	0,12	0
Mouse hepatoma	0,16	0	0,02	0

The distribution of the administered CA into the various bases of RNA was determined in another experimental series.

Healthy rats were given four consecutive injections of a CA solution, at intervals of 3 and 18 hours, the total dose being 25,000-28,000 cpm/g body weight. The animals were killed 2 hours after the last injection, the livers were removed and RNA was extracted, freed from protein and hydrolysed in 1.2 N $HClO_4$. The hydrolyzate was diluted with water and applied to a sheet of paper by means of a micropipette. The chromatogram was developed in ethanol/isopropyl alcohol/5 N HCl (2: 26:2 by Vol.). The spots were located in the ultraviolet light, cut out of the paper and eluted with 0.1 N HCl.

The purity of the eluted compounds was checked through their absorption spectra in the ultraviolet, using the SF-4 spectrophotometer. The eluated were transferred to aluminium planchets, evaporated, and the radioactivity determined as described above. No radioactivity was found in either adenine or guanine. In the pyrimidine bases the radioactivity was higher in uracil than in cytosine. In each mg of the RNA hydrolyzate the radioactivity of uracil accounted for 170 cpm, while that of cytosine was 100 cpm.

Pyrimidine Biosynthesis in the Tissues of Normal and Tumor-Bearing Animals

It was found (Table 3) that both at 2 hours and at 24 hours after labeled CA administration the intensity of label in both the nucleic acids and in the soluble fraction was higher in tumor-bearing animals than in normals. The difference became insignificant at 48 hours.

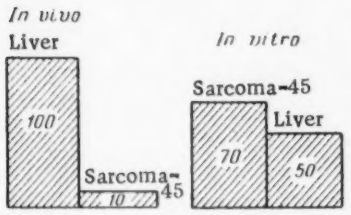
With mice bearing Ehrlich ascites the difference between the tumor-bearing and normal mice in the incorporation rate of CA was still evident at the 48-hour period. The radioactivity of the acid-soluble compounds and of the nuclei acids isolated from the tumor tissues was insignificant (Table 4), regardless of the type of tumor (solid or ascites).

The low level of label incorporation into tumor tissues was not due to the method of CA administration; the value remained on the same level whether the route was subcutaneous or intraperitoneal. Only after repeated doses, which brought about a saturation of the entire animal with the labeled compound, did the radioactivity of the

TABLE 5

CA Incorporation into the Nucleoproteins of Liver and Sarcoma-45 Slices in O_2-Saturated Media (in cpm/10 mg)

Tissue	O_2 saturation	Control
Sarcoma-45	78	18
Liver	59	13
Sarcoma-45	94	46
Liver	74	19
Sarcoma-45	60	40
Liver	48	20



Pyrimidine biosynthesis from CA in vivo and in vitro in liver and in sarcoma-45 (cpm/10 mg).

tumor tissues increase, and even in that case the values remained well below the corresponding values in the normal tissues. Thus, for instance, the incorporation of the label into the liver was about ten times more intensive than its incorporation into tumor.

The In Vitro Biosynthesis of Pyrimidines from CA

The experiments in vitro were carried out with slices of liver and sarcoma-45. Each vessel contained 0.5 g of the tissue slices and the labeled precursor equivalent to 100,000 cpm. The incubation was carried out with 4 ml of Krebs-Ringer, pH 7.2. The vessels were shaken at 37° for 90 min; the incubation was stopped by the addition of 0.2 N $HClO_4$. Further treatment was as described in "Methods".

It was found that, both with rat liver and sarcoma-45 slices, the incorporation of CA was considerably increased in the presence of an atmosphere O_2. The intensity of pyrimidine biosynthesis from CA in vitro was also compared with the corresponding process in vivo in a special series of experiments, using sarcoma-45.

It will be seen from Table 5, and from the figure, that in vitro the biosynthesis of pyrimidines from CA in sarcoma-45 exceeded that in liver slices, while in the intact animal, as already mentioned above, CA incorporation amounted to only one tenth of the intensity of this process in the liver.

DISCUSSION

The present results on the distribution of CA in the tissues and organs of normal and tumor-bearing animals are in agreement with the results of Anderson et al. [11] obtained with normal mice an of the strain CAF with a transplanted sarcoma-37. The behavior of CA in its incorporation into the nucleic acids may be compared to the behavior of another precursor of the nuclei acids pyrimidines, orotic acid [12,13]. Anderson et al. pointed out the very slight incorporation of CA into the DNA of liver and tumor. The present results are in good agreement with these observations.

Of interest is the present observation of the differences in the relative rates of CA incorporation into the pyrimidines of tumor tissues under in vitro and in vivo conditions. The relatively higher synthetic ability of the tumor tissue as compared to liver, under in vitro conditions, indicated the higher degree of viability of the tumor tissue and its adaptability to unfavorable conditions. It is also known that the intensity of protein synthesis (as measured by the incorporation of labeled amino acids) in vitro is higher in tumor tissue than in normal tissues under these same conditions, while the reverse holds in the intact animal [14].

It is possible that the low degree of CA incorporation into tumors in vivo is due to the fact that the main pool of CA, as well as of orotate, in the body is located in the liver; as a result of CA hydrolysis in the liver, only its breakdown products, with a considerably lower degree of label, may reach the tumor. The possibility of a lower permeability of the tumor tissues to CA must also be considered.

SUMMARY

Ureidocarbon-labeled carbamylaspartate was incorporated more intensively into the nuclei of liver and sarcoma-45 cells than into their mitochondria or supernatant fraction. Carbamylaspartate was found to be a precursor mainly of the RNA pyrimidine. It was shown that the incorporation of carbamylaspartate into the nucleic acids of tissues of tumor-bearing animals was more intensive than its incorporation into tissues of normal ones. Carbamylaspartate utilization by various transplantable tumors for nucleic acic pyrimidine biosynthesis in vivo was found to be low (about one tenth the corresponding rate of incorporation into liver). The situation was reversed in vitro.

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DETERMINATION OF BIOTIN WITH THE YEAST CANDIDA TROPICALIS

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Vitamin H or biotin in very small amount shows an activating action on the growth of microorganisms. For the determination of the biotin content in various materials a microbiologic method is used. The simplest such method is that in which yeast are used as the indicator. At first cultures of Saccharomyces cerevisiae were used [1-4]. In 1954 Shavlovskii [5] used a simpler method for determining biotin with Endomyces magnusii. Odintsova [6] recommended three types of yeast for determining biotin: Debaryomyces matruchoti, Zygosaccharomyces bisporus, and Zygosaccharomyces mongolicus.

The method presented here also uses yeast organisms.

Indicator Microorganisms

In a study of the use in vitamin production of a number of yeast taken from hydrolysis and sulfite-alcohol media, it was noted that the yeast C. tropicalis required only biotin among the vitamins. This characteristic suggested the use of such yeast for the determination of biotin. For comparison we took the well-known test organism Saccharomycodes ludwigii KM.

The yeast S. ludwigii KM requires for its growth the presence of five vitamins in the medium: thiamine, pyridoxine, biotin, pantothenic and nicotinic acids. This characteristic was used for working out microbiological methods for determining pyridoxine [7] and pantothenic acid [8]. We used this organism for determining nicotinic acid and biotin.

The yeast C. tropicalis CK-4, isolated from sulfite-alcohol spent wash, needs only biotin. The strain CK-4, in distinction from the other strains of C. tropicalis, grows in the form of single cells, rarely forming small branches and very rarely, pseudomycelia. Therefore it is easy to work with this strain, measuring the intensity of growth by cloudiness in a nephelometer or photoelectric colorimeter.

Extraction of Biotin. A sample of dry yeast (we used nutritional yeast) weighing 0.1-0.5 g, was ground to a powder in a coffee mill, treated with 10 ml of 6 N H_2SO_4, and hydrolyzed in an autoclave at 1 atmos for 1 hour. After autoclaving, the hydrolyzate was transferred to a calibrated flask with distilled water, neutralized with 6 N NaOH to pH 5.0-6.0, diluted to the mark, mixed and filtered through a paper filter. The dilution should correspond to 1 g of dry yeast in 6 liters of water.

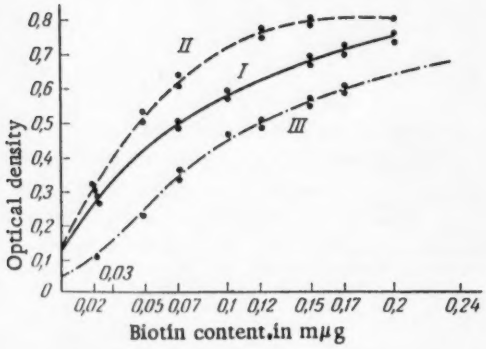
Technique of Determining Biotin. In the determination of biotin with C.tropicalis CK-4 no addition of any sort of vitamin to the nutritive medium is required. The Rieder medium* containing 1% glucose was placed in test tubes, 2 ml in each. From a standard aqueous solution of biotin which contained 10 μg/ml we prepared working solutions with a concentration of 0.2 mμ/ml and added them to the tubes with the medium in amounts of 0.1, 0.15, 0.23, 0.35, 0.5, 0.6, 0.75, 0.85, and 1.0 ml which corresponded to a biotin content of 0.02, 0.03, 0.05, 0.07, 0.1, 0.12, 0.15, 0.17, and 0.2 mμ g in the tubes.

* Composition of the medium: glucose 10.0 g; $(NH_4)_2SO_4$ 3.0 g; $MgSO_4$ 0.7 g; NaCl 0.5 g, $Ca(NO_3)_2$ 0.4 g, KH_2PO_4 1.0 g, K_2HPO_4 0.1 g, tap water 1 liter.

TABLE 1

Comparison of Three Methods for Determining Biotin Content of Biotin (in yeast extracts in mμ g/ml)

Method for determining biotin	Extract No.			
	1	2	3	4
S. ludwigii KM, medium with vitamins	0,15	0,3	0,14	0,4
C. tropicalis CK-4, medium without vitamins	0,15	0,29	0,16	0,38
C. tropicalis CK-4, medium with vitamins	0,10	0,33	0,21	0,42



Standard curve for determination of biotin. I) Indicator microorganism C. tropicalis CK-4, medium without vitamins; II) indicator organism C. tropicalis CK-4, medium with vitamins; III) indicator organism Saccharomycodes ludwigii KM, medium with vitamins.

In two tubes with medium placed 1 ml of water (blank samples). In the other tubes with medium we added the extracts under investigation, containing biotin in the amounts of 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 ml. In all the tubes we added distilled water to a volume of 3 ml, at first adding water to the tube with the medium and them the solution of vitamin or extract under investigation. If we used tubes of the same diameter for the determination, then in parallel tubes, the intensity of growth agreed perfectly; in such a case it was enough to use only two tubes for each concentration of biotin. All the tubes were sterilized in an autoclave at 0.5 atmos for 15 min and after cooling were seeded with a two day old culture of C. tropicalis CK-4 grown on spent wash-agar (one loop of culture was seeded into 10 ml of sterile tap water; seeding was carried out with a pipet, one drop in each tube). Incubation was carried out at 32-36°; the intensity of growth was measured after 40-48 hours in a photoelectric colorimeter with a green filter in a cuvette 5 mm across.

For determination of the value of the optical density of the biotin solutions we constructed a standard curve on which we determined by interpolation the biotin content of the extracts which we then calculated over for the culture and for the absolute dry weight of substance.

The determination of biotin with S. ludwigii KM was carried out in exactly the same way as with C. tropicalis CK-4, but the following vitamins were added to the Rieder medium: for each liter, 1 mg of thiamine, pyridoxine, and nicotinic acid, 3 mg of inositol, 0.02 mg of pantothenic acid. The other vitamins of the B group contained in the yeast extract for this culture, when used for the determination of bitoin, actually had no effect on the growth of C. tropicalis CK-4 in the medium without vitamins. We tested the effect of adding vitamins to the medium for C. tropicalis CK-4 in the same amounts as were used for S. ludwigii KM.

The figure shows the standard curve for determining biotin with S. ludwigii KM and C. tropicalis CK-4 on a medium with vitamins and also with C. tropicalis CK-4 on a medium without vitamins.

The addition of the other vitamins to the nutrient medium stimulates the growth of C. tropicalis CK-4.

Method of Extraction of Biotin from the Material under Study

Filippov [4] suggested carrying out fractional extraction of biotin by triple hydrolysis of the material in an autoclave with 1 N H_2SO_4. On the basis of our results, we consider that in order to obtain an extract from yeast it is not necessary to carry out a fractional extraction, as the following experiments show.

TABLE 2

Effect of Sulfuric Acid Concentration and Duration of Hydrolysis on Yield of Biotin from Yeast (biotin content in μg/g in absolutely dry substance)

H_2SO_4 concentration	Duration of hydrolysis	Yeast sample I	Yeast sample II
1 N	30 min	0,61	1,0
	1 hour	0,72	1,46
3 N	30 min	0,73	1,14
	1 hour	0,88	1,28
6 N	30 min	1,29	1,48
	1 hour	1,40	1,47
9 N	30 min	1,37	1,45
	1 hour	1,25	1,30
12 N	30 min	1,41	1,33
	1 hout	1,36	1,25
15 N	30 min	1,12	1,91
	1 hour	0,93	0,88

TABLE 3

Effect of Autolysis and Acid Hydrolysis of Yeast on Biotin Yield (biotin content in μg/g on absolute dry weight)

Type of yeast	Autolysis	Hydrolysis with 6 N H_2SO_4
Glucose synthetic medium	0,716	1,356
Cane hydrolyzate	0,613	1,120

Two samples of the same type of yeast were hydrolyzed with 1 N H_2SO_4 in an autoclave. One sample was centrifuged each hour, the liquid was poured into a calibrated flask, and the precipitate was again treated with the same solution of H_2SO_4 and again autoclaved; this operation was repeated three times (fractional extraction according to Filippov). The second sample was hydrolyzed all three times along with the first, but the sulfuric acid was not replaced (simulated Filippov extraction). The biotin content found by both methods was approximately the same; no decomposition of biotin was found in the simulated method.

Biotin content (μg/g) in absolute dry substance	
Fractional extraction	Simulated extraction
1,72	1,9
0,669	0,79

Hence we can say that the more difficult fractional extraction is not needed.

We also carried out an investigation of the effect of concentration of sulfuric acid and length of hydrolysis on yield of yeast biotin. Hydrolysis was carried out in an autoclave at 1 atmos; yeast samples from 0.2 to 0.5 g in all cases were added to 10 ml of H_2SO_4 solution. The results of the experiments show that with increasing concentration of sulfuric acid, the biotin yield increases. Autoclaving with 6 N H_2SO_4 at 1 atmos for an hour gives the greatest biotin yield. Decomposition of biotin is found when the concentration is very much greater, that is, at 15 N H_2SO_4 (Table 2).

We also studied the effect of autolysis on the liberation of biotin. The results of the experiments (Table 3) showed that in autolysis of yeast half as much biotin is liberated from protein complexes as by hydrolysis.

SUMMARY

The suggested method for determination of biotin using C. tropicalis CK-4 in a Rieder medium without addition of vitamins is comparatively simple and permits application without addition of other vitamins. If there is no apparatus for measuring the change in intensity of growth by cloudiness, we can determine the growth of the yeast by the weight of the cultures. For this it is necessary to use the Rieder medium with 2% glucose and pour 10 or 20 ml of it into a small calibrated flask. The mass of yeast which forms can be filtered through a glass filter (No. 4) and also through a membrane or through a paper filter first dried to constant weight. In this case it is best to use a strain of C. tropicalis as an indicator which forms branches and aggregates of a large number of cells and can be filtered easily. Such are strains CK-3 and CK-5 which are also sensitive to the presence of biotin in the medium obtained from sulfite-alcohol factories.

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NITROGENOUS COMPONENTS OF LIPIDS IN THE LIPOPROTEINS OF PLASTIDS

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Phospholipids are widely distributed in various tissues of the organism and accumulate particularly intensely in tissues which are characterized by intensive metabolism. However, the role of phospholipids in cell metabolism is far from being fully explained. The possibility that phospholipids share actively in metabolic reactions arises because of their bonds with protein molecules. Actually, phospholipids are found in the composition of biologically active proteins—in cytochrome oxidase and in cytochrome [1].

Among the nitrogenous bases of phospholipids, along with ethanol amine and choline , serine has been found at first in brain tissue and then generally in the organism. In recent years a number of studies have appeared which indicate the presence of other amino acids or peptides in phospholipid composition. These peptides differ somewhat in their own amino acid composition one from another, but serine is always present in them.

Lipopeptides were discovered in the blood [2,3]. Thus, Macheboeuf and co-workers [3] discovered amino acids, including serine, along with ethanol amino as nitrogenous components of blood lipids. However, the quantity of serine did not exceed the content of the other amino acids. The authors suggested that the compounds which they isolated were lipopeptides. Further, Rouhi [4] showed that in vitro among various classes of lipids only the phospholipids could combine with amino acids, peptides, and proteins.

In the phospholipids of animal and human tissues amino acids have been found [5,6]. It was established that amino acids were present in the electrophoretically homogeneous groups of lipids from human liver and animal lungs. Hakomori [7] showed that ethanol amine combined through its amino group with the carboxyl group of amino acids and that glycine had a free amino group. It should be noted that before these studies , the peptide bond in sphingophosphatides between fatty acids and the amino group of sphingosine was the only known case of a peptide bond in the lipids. The work of Hakomori is certainly of interest since it throws light on the structure of phospholipids which have been little studied and thus permits a closer approach to an understanding of their role and mechanism of action in the organism.

Rosenberg and Chargaff [8] observed in cattle brain a whole group of amino acids in the composition of the complex mucolipids, inhibitors of blood coagulation. The lipids were homogeneous in the ultracentrifuge and in electrophoresis. The amino acids composed less than 5% of the total weight of the molecule. They could be detected after hydrolysis by hydrochloric acid. The amino acids could not be removed by various methods of purification, including paper electrophoresis. The authors concluded that in the complex lipids glycolipids occurred in chemical combination with polypeptides. The amino acids in the polypeptides were not identified.

Westley, Wren, and Mitchell [9] studied the nitrogenous components of phospholipids from different tissues of rats, yolks of eggs, drosophila and Eremothecium ashbyii. After hydrolysis by hydrochloric acid, ethanol amine and amino acids were detected in the hydrolyzate. The authors assumed that the structure of the nitrogenous compounds which they found was that of a peptidyl-phosphotidylethanol amine.

All these studies concerned the animal organism. Amino acids as the nitrogenous components of phosphatides in plants have been little studied. Schulte and Krause [10] found in the phosphatides of wheat and rye germ, besides choline and ethanol amine, alse serine and another compounds closely related to it. Both serine and the

unknown compound were combined with phosphatides and were not contaminants, as was shown by purification of the phosphatides by dialysis and paper electrophoresis. The unknown compound contained an amino group. We suggest on the basis of the results of Schulte and Krause that the ninhydrin-positive spot on the chromatogram could come from a peptide, since the authors carried out hydrolysis with 2 N H_2SO_4. Under these conditions not all peptides are split into amino acids.

In plant cells the plastids are the objects in which a considerable part of the enzymatic activity is centered [11]. Chloroplasts isolated from the cells can synthesize proteins and lipids [11-13]. In chemical composition the plastids are rich in proteins and lipids. Phospholipids are found among the lipids [14]. Some of these are easily extracted by organic solvents, others with difficulty, since they are combined with proteins.

The composition of the plastid phosphatids and their structures have been litle studied. We have isolated phospholipids from the leucoplasts of sugar beet root [15]. Among their nitrogenous components we have found the amino acids: glutamic and aspartic acids, glycine, alanine, and serine. The isolated phospholipids were combined with the plastid proteins. The work of Kauffman and Ködding [16] followed ours. The authors isolated from the chloroplast walls of spinach leaves an ether soluble compound which contanined amino acids. This substance was a glycerophosphatide. After 8 hour hydrolysis with 20% HCl, they found in the hydrolyzate glycerol fatty acids, H_3RO_4, choline, and ethanol amine, and after chromatographic separation, a small amount of glycine, alanine, serine, and dicarboxylic amino acids.

On the basis of the experimental data the authors suggested that the combination of the amino acid with the phosphoric acid occurred through the carboxyl groups as a high-energy acylphosphate bond. They judged the presence of the acylphosphate bond by the fact that the amino groups of the amino acids mentioned were free. The rate of hydrolytic splitting of amino acids heated with water correspond to the rate of splitting an acylphosphate bond. Further, Kauffman [16] noted in chloroplast walls of various dicotyledinous plants, nettles, spinach, sugar beet, and sunflower, amino acids not firmly bound to proteins.

In our previously published work we showed that the amino acid composition of lipoproteins isolated from plastids did not remain constant during ontogenesis [17,18]. The content of one amino acid remained constant while the amount of another changed to a great extent. In particular, in the lipoproteins isolated from the leucoplastids of sugar beet roots, the amount of serine fell with age of the plant (from 12.6% in young roots to 5.8% in mature ones). The content of amino acids into which serine could be transformed in the organism, namely, cystine, glycine, and threonine, increased by the same value (by 7.0%).

When we consider that serine is an important link in the chain of many metabolic reactions in the organism and shares in amino acid and phosphatid metabolism, we find that these data indicate the reorganization of the protein molecule and the participation of serine in this reorganization.

The question arises whether serine is present in the plastid lipids combined with proteins and whether this compound shares in the reorganization of the protein molecule.

The results of the study cast light on the first part of the question. We have tried two method for its solution. We have studied lipoproteins isolated from the leucoplastids of sugar beet root and from the chloroplasts of bean leaves. Also we have isolated phospholipids from the same plastids and from wheat seeds by fractionation with organic solvents. We have studied the nitrogenous components of the phosphatides.

EXPERIMENTAL PART

Materials. The objects of our study were the leucoplastids of roots of sugar beets of the "Verkhnyachka" variety and chloroplasts of the leaves of beans of the "Latvia" variety. The sugar beets were grown on an experimental plot. We used the roots of mature plants which had been kept for several months. The beans were grown in a greenhouse and two-week-old plants were used for the experiments. The seeds of summer wheat of the "Artemov" variety (V. lutescens) and the "Narodnaya" variety (V. hordeiforme), 1957 harvest, were obtained from the Ukrainian Institute for Plant Culture, Selection, and Genetics. For analysis we used completly ground flour obtained with an ordinary laboratory mill.

Separation of plastids. The plastids were isolated from the remaining sap by grinding the beet roots or bean leaves in 0.5 M sucrose. Isolation was carried out in a Sharpless supercentrifuge as had been described previously [18,19]. All the operations were carried out at a temperature of 1-3°.

Removal and fractionation of the lipids from the plastids or from the wheat seeds was carried out by the method described by Delsal [21] for blood lipids. The lipids were removed with a mixture of methylal-methanol (4:1) and then with methanol. Theproteins then remained in the precipitate (1). Water and methanol were then added to the methylal-methanol solution in an amount to bring the concentration of the methanol to 66%. Then the glycerol and steroids were removed with light ligroin (boiling to 60°). The organic solvent was distilled in a vacuum from the solution of methylal-methanol which contained the phospholipids in a stream of nitrogen at room temperature. Ethyl alcohol was added to the remaining water solution to a concentration of about 30% and the phospholipids were removed with ligroin which was then distilled off in a stream of nitrogen.

The resulting fraction, composed of free phospholipids, will from here on be called "free PL". Precipitate I which after removal of free PL contains proteins and the lipids combined with them was treated with 96% ethyl alcohol with heating to boiling for two hours. Then the alcohol extracted was separated from the precipitate (II) and evaporated dry in a vacuum. The dry residue and also precipitate II were extracted with methylal-methanol and fractionated by the method described. The phospholipid fraction contained phospholipids combined with protein and was called "bound PL".

The phosphorus content in the glyceride fraction consisted of traces up to 0.06%. The phospholipid fraction obtained from root leucoplasts contained 1.8-2.0% P on the dry weight, and that isolated from wheat seeds, 1.6%. Determination of phosphorus was carried out according to Weil [22]. The distribution of phosphorus in the fractions showed the possibility of applying the method of Delsal to plant materials.

Isolation of lipoproteins and separation of lipids from proteins. The lipoprotein was separated from the leucoplasts of sugar beet roots and from the chloroplasts of bean leaves by the method which we had previously worked out [23]. It consists in removing the protein from the plastids with 75-77% ethyl alcohol acidified by hydrochloric acid to pH 3. The alcohol solution was evaporated in a vacuum. The residue was washed repeatedly with water to remove the α-naphthol reaction for carbohydrates, and was then dissolved in an acid medium from which the lipoproteins were precipitated by addition of alcoholic NaOH solution to pH about 6. Solution and precipitation were repeated not less than three times. The preparation was dried lyophilically. The entire operation was carried out at 0-3°. The protein preparations isolated from the leucoplasts and chloroplasts contained about 11.4-10.6 % nitrogen on the dry weight when determined by Kjeldahl and Dumas micromethods.

For separation of the lipids from the lipoproteins, the preparation was heated with 96% ethyl alcohol on the boiling water bath for 2 hours. The solution was separated from the residue I by centrifuging. After the alcohol was removed by evaporation on the water bath, possible contaminating proteins were removed from the solution by precipitation with 5% TCA. After centrifuging, we removed the TCA with ether and evaporated the water portion to dryness (residue II). Residue I was further extracted with a light ligroin fraction. The extracts were evaporated on the water bath and combined with residue II. The preparation before and after hydrolysis was analyzed by electrophoresis and paper chromatography.

Hydrolysis of the preparations in all cases was carried out with 6 N HCl at 103-105° for 22-24 hours. Hydrochloric acid was removed in a vacuum desiccator over NaOH.

Electrophoresis was carried out on a strip of Whatman No. 1 or No. 3 filter paper 6 cm wide and 36 cm long. The electrophoresis apparatus was constructed of glass and plexiglas according to the design of Durham as modified by Macheboeuf et al. [24] with some changes. The changes concerned the complex labyrinth in which the platinum electrodes were placed. For running the electrophoresis with evaporation [24] the electrophoretogram was placed under a glass cylinder covered with a sheet of asbestos on which stood a cuvette with a cooling mixture for better evaporation. The evaporatingsolution soaked into the asbestos sheet.

Conditions for electrophoresis : voltage 450 v, current strength 0.612.0 ma; electrolyte, veronal buffer, pH 8.6 or 0.5 M acetic acid, pH 2.3 . The substance was placed on the paper as a narrow strip. When we used an acid buffer the substance was not placed in the middle of the paper strip, but 4-6 cm closer to the anode. Electrophoresis was carried out for 6-7 hours at room temperature.

Chromatographic analysis. For the identification of the amino acids we ran a two-phase paper chromatogram on Schleicher-Schull 2043b paper, using two systems of solvents [25]. The first system : phenol-collidine- α -picoline (3:2) saturated with water; the second : in the first direction, a mixture of methanol-water-pyridine (40:10:2) , in the second, n -butanol-methylethyl ketone-water-diethylamine (20:20:10:2). In some cases the solvents which

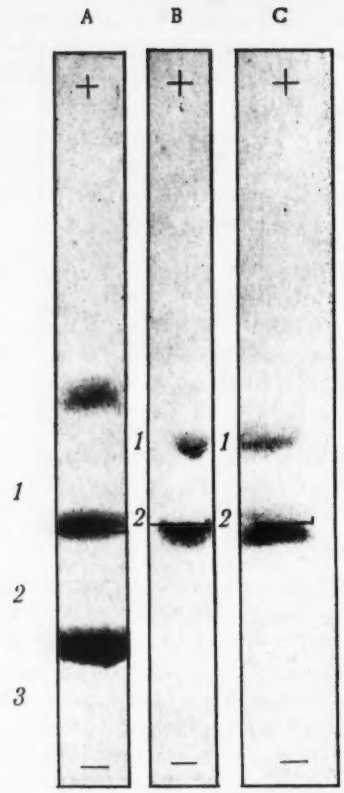

Fig. 1. Amino acids combined with protein in plastid phospholipids. Electrophoresis at 400 v and 1.8 ma, duration 3.5 hours in a veronal buffer, pH 8.7. A) Control; B) hydrolyzate of phospholipids separated according to Delsal; C) hydrolyzate of lipid portion of lipoproteins. 1) Aspartic and glutamic acids; 2) serine, glycine, alanine; 3) lysine.

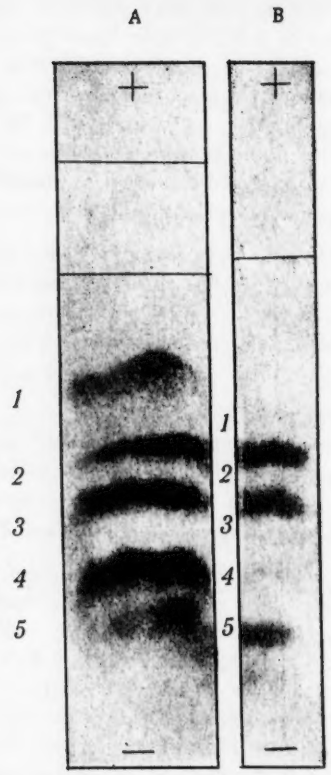

Fig. 2. Amino acids bound with protein of plastid phospholipids. Electrophoresis at 450 v, 1.0 M acetic acid, pH 2.3, duration 6 hours for A and 5 hours for B. A) Control; B) hydrolyzate of phospholipids separated according to Delsal. 1) Aspartic acid; 2) glutamic acid; 3) serine; 4) alanine; 5) glycine.

contained the studied substances were first submitted to electrophoretic separation. The zones obtained on the electrophoretogram were eluted into a one-phase chromatogram and the amino acids were identified by the chromatographic method in phenol saturated with water. Pure preparations of the corresponding amino acids served as standards.

Coloring the substances on the electrophoretograms and chromatograms. The lipoproteins were colored by brom phenol blue on the protein [26]. The lipids bound to the protein were colored with Sudan black by the method of McDonald and Marback [27]. The amino acids were colored with a 0.5 % ninhydrin solution in acetone applied to the paper. The color developed at room temperature. The color of the resulting spots was then fixed in a a solution of copper nitrate.

RESULTS AND DISCUSSION

Lipids separated from the plastid lipoproteins and also the phospholipids bound to the proteins obtained by fractionation in organic solvents contained before hydrolysis only one ninhydrin-positive compound which occupied an indefinite position on the two-phase chromatogram. In hydrolysis this compound split into several amino acids.

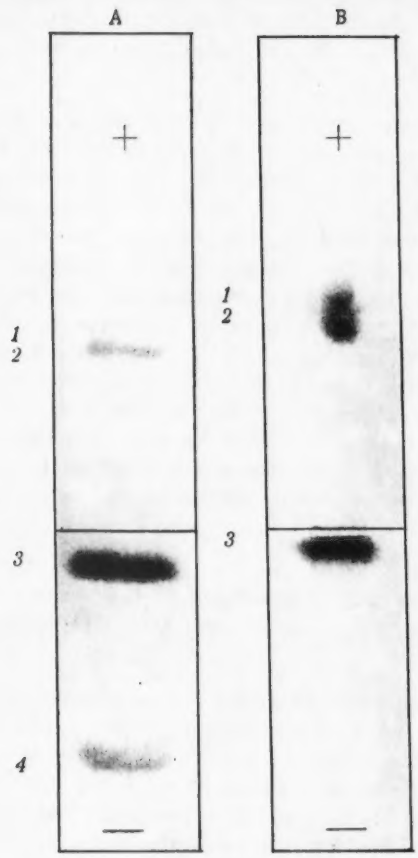

Fig. 3. Amino acids of phospholipids bound with protein, from wheat seeds. Electrophoresis at 400 v, 1.8 ma, duration 6 hours in a veronal buffer, pH 8.4. A) Control; B) hydrolyzate of phospholipids separated according to Delsal. 1) Aspartic acid; 2) glutamic acid; 3) glycine, serine, alanine; 4) lysine.

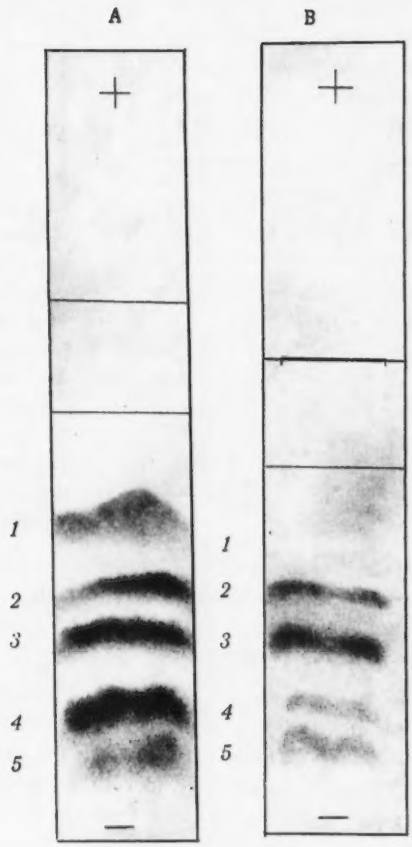

Fig. 4. Amino acids of the lipid portion of lipoproteins. Electrophoresis at 450 v, 1.0 ma, in 0.5 M acetic acid, pH 2.3, duration 6 hours. A) Control; B) hydrolyzate of plastid lipids bound with protein. The lipoprotein was purified by electrophoresis. 1) Aspartic acid; 2) glutamic acid; 3) serine; 4) alanine; 5) glycine.

Electrophoresis at pH 8.6 showed that the substance contained dicarboxylic and neutral amino acids; basic amino acids were not found (Fig. 1). Electrophoretic separation at pH 2.3 gave five ninhydrin positive zones (Fig. 2). By elution and later chromatography in phenol, five amino acids were identified: aspartic and glutamic acid, serine, alanine, and glycine. Thus, we determined partially the amino acid composition of the nitrogenous components of the lipids from bean chloroplasts.

In two-phase chromatography of the hydrolyzate the ninhydrin-positive compounds of the sugar beet root leucoplasts were identified as the same five amino acids: aspartic and glutamic acids, serine, alanine, and glycine. From the phospholipids of the wheat seeds we isolated fractions by organic solvents according to the Delsal method. In the phospholipids bound with protein, after hydrolysis, we found aspartic and glutamic acids and neutral amino acids (Fig. 3). Electrophoresis at pH 2.3 showed the presence of five ninhydrin-positive zones.

The amino acids were not chance contaminants in the phospholipids or the lipoproteins. Aside from the purification of the preparations described above, we used paper electrophoresis, for the same purpose when they were separated. Electrophoresis was carried out at pH 2.3. For this purpose the protein solution was quickly deposited from a micropipet on the electrolyte moistened paper and placed in the apparatus. Electrophoresis was

TABLE 1

Amino Acid Content in Lipoproteins Isolated from Leucoplasts of Sugar Beet Roots and Bean Leaf Chloroplasts (in % dry weight)

Amino acids	Leucoplasts		Chloroplasts
	lipoproteins	other proteins	lipo proteins
Aspartic	1,8	1,4	3,1
Glutamic	4,3	6,2	8,2
Glycine	6,1	4,1	3,5
Serine	5,8	3,9	9,3
Alanine	5,8	5,6	5,4
Leucine	5,9	4,3	15,3
Arginine	18,1	14,9	6,3
Lysine	8,2	11,1	9,3

carried out without evaporation in a moist atmosphere. Under these conditions the lipoprotein separated from the leucoplastids moved far toward the cathode from the original position (isoelectric point of the protein about 6).

The electrophoretogram was cut lengthwise into three strips: the zones of protein compounds on the strips gave a color with brom phenol blue, with Sudan black, and weakly, a slowly developing lilac color with ninhydrin. At the starting point there was always a considerable amount of remaining substance which gave the same reactions. We assumed that the spot at the starting position is the same lipoprotein, denatured during deposition on the paper as a result of some drying and adsorption by the paper. In order to show this, in special experiments (experiments with evaporation) the lipoprotein solution was deposited on paper not moistened by the buffer and without conditions in which part of the protein avoided denaturation. After electrophoresis in these experiments the whole substance remained at the starting position. The portion of the paper toward the cathode was free both of proteins and of ninhydrin positive compounds.

Further, in experiments 1 and 2, all the zones of the electrophoretogram which contained lipoprotein were cut into pieces and separately treated with boiling alcohol, The lipids were removed from the solution and purified from possible protein contaminants as described above. After hydrolysis the same five spots were found in the lipid preaparations in both experiments.

The preparations of bound lipids isolated from the plastids fractionated in organic solvents according to Delsal were also purified by electrophoresis at pH 2,3 from possible protein or peptide contaminants. Under these conditions the phospholipids were adsorbed by the paper and remained at the starting position, while amino acids and peptides migrated toward the cathode; thus, we could fully purify the phospholipids by paper electrophoresis from amino acids artificially mixed with them [9]. However, in our experiments ninhydrin treatment showed no colored zone in the cathode portion of the electrophoretogram. Amino acids were not separated even when the phospholipids were eluted with ligroin. They were found in the hydrolyzate residue after removal of the solvent (Fig. 4). All these experiments confirm that amino acids (or peptides) are not contaminants in the preparations.

It might be suggested that in our experiments the coloring by ninhydrin is due to slight contamination by proteins in the lipid preparation which are not precipitated by trichloroacetic acid. However, the fact that lipoproteins from sugar beet leucoplasts are rich in basic amino acids and comparatively poor in aspartic and glutamic acids, serine, and alanine, and the bean protein [20] is rich in leucine and poor in aspartic acid, glycine, and alanine (Table 1) should exclude such a possibility.

In Table 1 we give the amount of some amino acids in the lipoproteins isolated from sugar beet leucoplasts [18,17] and from bean leaf chloroplasts [20] . If there were contamination of the phospholipids by components of the lipoproteins basic amino acids in the first case and leucine in the second would certainly be colored by ninhydrin. However, as Fig. 1 shows, basic amino acids were not found in the preparations studied. Leucine was also absent from the nitrogenous components of bean phospholipids, as chromatography showed.

It can also be suggested that the ninhydrin positive compounds are residues of other proteins picked up by the lipoproteins during their isolation. In Table 1 (column 2) we give the content of the amino acids which interested us in the proteins of sugar beet leucoplasts which are precipitated along with the rest from the acid alcohol extract [17]. As the results in the table show, the proteins also contain many basic amino acids and not much aspartic acid, glycine, or serine. Thus the doubt which we have expressed concerning the contamination by proteins of the lipid preparations is unfounded. The possibilities have also been tested by experiments in which the phospholipids were obtained by fractionation in organic solvents according to Delsal.

Kauffman and Kõdding [16] showed the presence of amino acids in phosphatides of spinach leaf chloroplasts. The labile bond of the amino acids with the phosphatides and also the presence of the same amino acids which were first observed in photosynthesis [28] led these authors to suggest that the phosphoaminolipids took part in

TABLE 2

Composition of the Nitrogenous Components of Lipids

Materials	Amino acids	Authors
Plant materials		
Leaf chloroplasts		
bean	glyc., asp., ser., glut., al.	Our data
spinach	glyc., asp., ser., glut., al.	Kauffmann, Kodding[16]
sugar beet	glyc., asp., ser., glut., al.	" "
sunflower	glyc., asp., ser., glut., al.	" "
nettles	glyc., asp., ser., glut., al.	" "
Leucoplasts of sugar beet roots	glyc., asp., ser., glut., al.	Our data
Wheat seeds	glyc., asp., ser., glut., al.	Our data
Animal materials		
Hog lung	glyc., asp., ser., glut., al., arg.	Hirata [6]
Human liver	$-H_2N$-glyc.,ser., glut. (-CO)ethan.	Hakamori [7]
Rat liver	amine asp.,ser., glut., cys.	Westley et al. [9]
Rat brain	asp., ser., glut.	"
Egg yolk	asp., ser., glut.	"
Drosophila,	asp., ser., glut.	"
Eremothecium ashbyii	asp., val., leuc.	"
Beef brain	peptide	Rosember et al. [8]
Cytochrome oxidase	peptide	Marinetti el al. [1]

TABLE 3

Composition of Peptides Which Contain Active Serine in Enzymes

Enzyme	Amino acids	Authors
Chymotrypsin	-glyc-asp-ser-glyc-glut-prol-leuc-	Neurath, Dixon et al. [29]
	-glyc-asp-ser-glyc-glut-al	Turba, Gundlach [30]
Trypsin	-glyc-glyc-asp-ser-glyc-prol-val	Neurath, Dixon et al. [29]
Thrombin	-glyc-asp-ser-glyc-	Gladner, Laki [31]
Phosphoglucomutase	-glyc-asp-ser-glyc-	Koshland, Erwin [32]

photosynthesis. The authors considered this to be the more likely since this labile bond was found in the chloroplasts. However, the same amino acids were observed by us in root leucoplasts. The amino acids were not split from the lipids by boiling with aqueous ethyl alcohol. We found them only after hydrolysis with 6 N HCl. Thus we must assume that the amino acids are firmly bound to each other (possibly in the form of peptides).

In the modern literature there is already sufficient evidence as to the presence of peptides as nitrogenous components of phospholipids of blood and various animal tissues, so that the presence of peptides in plant lipids is not unexpected. The relatively constant composition of the peptides in different materials is noteworthy. Only some of the amino acids vary,while serine, glycine, and one of the dicarboxylic amino acids are always present. Often there are two amino acids and alanine. In Table 2 we give the amino acids found in the nitrogenous components of some phospholipids. In Table 3 we give the results of the analysis of peptides united to active serine in some enzyme proteins. This shows that these peptides contain the same group of amino acids. Among such enzymes are proteolytic ones.

Fraenkel-Conrat and Singer [33] isolated a highly purified infectious ribonucleic acid, capable of reproducing tobacco mosaic virus. After hydrolysis of the preparation, they found aspartic and glutamic acids and threonine. The assumption that these amino acids belonged to a contaminating mixture of virus protein was unlikely, as the authors showed, since it the virus protein other amino acids predominated.

These results raise the question of the possible role of amino acids of this group in the formation of proteins by ribonucleic acids.

We found amino acids in the composition of lipids not only in the phospholipids of leaves. They were also found in the phospholipids of beet roots and wheat seeds. The latter fact of the greater interest because the amino acid composition of seed proteins changes during their sprouting. Thus, the original nitrogenous components the amino acids, in plant phospholipids apparently show general regularities.

The results which we have presented allow us to suggested the participation of the nitrogenous components of the phospholipids bound to protein, in metabolic reactions of the plant cell, and especially in the formation of the protein molecule.

SUMMARY

We have studied the nitrogenous bases of lipids bound to proteins in leaf chloroplasts and root leucoplasts, and also the nitrogenous bases of wheat seed phospholipids.

Among the nitrogenous bases we have found ninhydrin positive compounds which split up after hydrolysis into amino acids; we have identified in the hydrolyzate serine, aspartic acid, glutamic acid, glycine, and alanine. Some results lead us to believe that the ninhydrin positive compounds are peptides. The peptides (or amino acids) are not contaminants but enter the composition of the lipid portion of the lipoprotein.

We have suggested that the phospholipids combined with proteins share actively in the metabolic reactions of the plant organism.

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THE EFFECT OF IONIZING RADIATION, ADRENALECTOMY AND CORTISONE TREATMENT ON THE INTESTINAL ABSORPTION OF AMINO ACIDS

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Irradiation with x-rays is known to inhibit digestion. It has been established [1] that x-irradiation caused the retardation in the alimentary tract of dioleylpalmitin-C^{14}, the degree of retardation being directly related to the irradiation dose. This retardation in evacuation is apparently due to a decreased absorbing ability of the intestinal wall. There are published data on the observed inhibition by x-irradiation of the intestinal absorption of sugars [2,3] and metal ions [4]. The information on the effect of x-irradiationon the intestinal absorption of amino acids is limited. It is known that the absorption of methionine [5] and glycine [6] from the intestinal tracts is depressed by irradiation in dependence on the dose delivered.

It is also known that the processes concerned with oxidative phosphorylation are inhibited by x-irradiation. Irradiation was shown to cause the uncoupling of oxidation from phosphorylation [7-10] and a fall in the incorporation of P^{32} into DNA and RNA [11]. Phosphoprotein phosphatase activity in the small intestine was also shown to fall following irradiation [12]. Heavy x-irradiation was shown to decrease the activity of the enzymes concerned with transamination in the small intestine of the rat [13]. A similar decrease in the activity of these enzymes was reported to occur as a result of adrenalectomy, but could be versed by the administration of cortisone. The similar nature of the changes evoked by radiation and by adrenalectomy, and the ability of cortisone to counteract these changes suggested the possibility of interference with the hormonal activity of the adrenal cortex during irradiation. It is known that the adrenal cortical hormones are also active in the processes of oxidative phosphorylation. The suppression of glucose [14-16] and of fatty acids [17] absorption observed in adrenalectomized animals can also be alleviated by treatment with cortisone, and particularly with DOC (desoxycorticosterone). Cortisone brings about an increase in esterified phosphorus in liver and muscle with a concomitant decrease in inorganic phosphate [18,19]. The favorable effect of adrenal cortical extracts and cortisone administration on carbohydrate assimilation, as demonstrated in clinical cases, may be due to the activation of intestinal phosphorylating mechanisms, which have been disturbed as a result of adrenal hypofunction [20].

It was also shown that glycine absorption was inhibited in adrenalectomized rats [21].

Published data thus indicate that x-irradiation inhibits the absorption of the majority of compounds in the intestine, and that it also interferes with oxidative phosphorylation. It is thus feasible that the radiation-induced suppression of absorption is related to the failure of oxidative phosphorylation, on the basis of which possibly lies adrenal disfunction.

In view of the previously established [22] dependence of intestinal absorption of amino acids on phosphorylation, it was of interest to investigate the effect of x-irradiation, adrenalectomy and cortisone treatment on the intestinal absorption of amino acid mixtures and on protein assimilation.

EXPERIMENTAL PART

The work was carried out in two stages. The first stage consisted of a study of the assimilation of protein nitrogen in rats kept on a complete synthetic diet with casein as a nitrogen cource. The animals were divided into

The Effect of Total Body Irradiation and Cortisone Administration on Certain Denominators of Protein Metabolism in the Rat (means of 5 experiments; controls from 10 experiments)

Group of rats	Protein Assimilation, %	Nitrogen in 24-hr urine	N balance, %	Intestinal wall content of	
				N, mg/g tissue	Labile P, mg/g tissue
Controls	92	0,24	0,28	20	0,75
3 days post-irradiation	90	0,1	0,23	21	0,5
14 days post-irradiation	91	0,11	0,2	21	0,5
cortisone-treated	89	0,31	0,1	12	0,73

three groups: the first, control, untreated; in the second group the animals received total body irradiation of 400 r; the animals of the third group received daily injection of cortisone acetate, 10 mg each, for one week. In all three groups the assimilation of protein nitrogen and labile phosphorus content of the intestinal wall were determined. Nitrogen was determined, after ashing, according to Conway, and labile phosphorus was estimated after preliminary precipitation of the inorganic phosphate with magnesia mixture. The experiments in the first stage involved 25 rats.

The second stage consisted of a study of the intestinal absorption of a mixture of amino acids and of histidine The small intestines in these animals were ligated at both ends under local anesthesia (novocain) and the amino acid mixture (2 ml of a 12-14% solution) was injected into the lumen with the aid of a syringe. The amino acid mixture was injected alone, or with the addition of ATP, phosphate and magnesium salt. As a rule, 40 mg of ATP, 20 mg of phosphate and one mmole of Mg^{++} as $MgCl_2$ was given per rat. All essential amino acids were present in the mixture. The nonessential amino acids in the mixture comprized glycine, alanine, glutamate, serine. Before injection the mixture was adjusted to pH 7. One hour after introduction of the mixture the contents of the intestinal section (washed out four times with water) were tested for nitrogen and amino acid content. The intensity of amino acid absorption was determined from the difference between the amount introduced and that remaining after the precipitation of proteins with TCA. Quantitative estimation of amino acids was carried out by mineralization followed by the Conway procedure, and also independently, using ninhydrin. Histidine was determined according to Meshkova [23].

Four groups of animals were used in the second stage: the first, control group, was untreated; the animals of the second group were irradiated and the effect of radiation on the absorption of amino acids at various time intervals postirradiation was studied; in the third group the effect of adrenalectomy on the absorption of amino acids was studied, while in the fourth the corresponding effect of cortisone administration was followed. Eighty rats were used in this series.

RESULTS

It was found from the control experiments, in the first stage, that in intact rats protein assimilation varied within the limits of 91-94%; nitrogen balance was 0.2-0.28 g and the nitrogen and labile phosphorus content of the intestinal wall was 18-22 and 0.5-1.0 mg/g, respectively.

There have been no significant changes in the variables studied in the group of animals which received x-irradiation (400 r of TBR). Shown in Table 1 are the results obtained on the third and fourteenth day postirradiation. Irradiation caused a decrease in food consumption, a slight decrease in assimilation of dietary nitrogen and a decrease in urinary nitrogen excretion. The nitrogen content of the intestine remained unaltered, and in some cases there was a decrease in the labile P content of the intestinal wall. Thus, the dose of radiant energy delivered (400 r), failed to show any effect, compared to controls, at the particular postirradiation periods investigated.

Cortisone-treated animals suffered considerable loss of weight and showed a somewhat decreased assimilation of protein and low nitrogen balance.

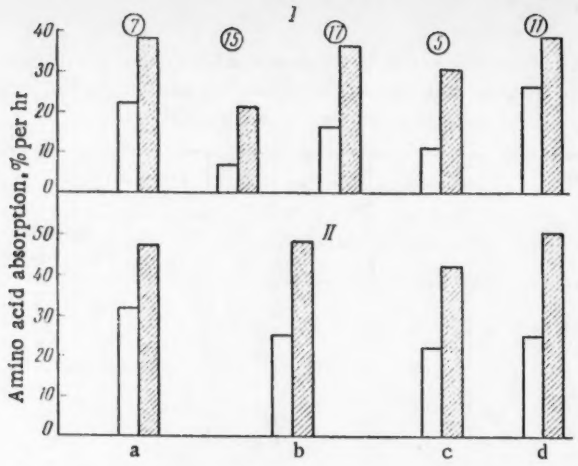

Fig. 1. The absorption of amino acid mixtures in irradiated rats (500-600 r) (I) as compared to normals (II). Numbers in circles refer to days postirradiation. Unshaded columns — amino acid mixture without additions; shaded columns — with added ATP, phosphate and Mg^{++}. a) 9% amino acid solution; b) 12% with added myol* preparation; c) 14% solution. Each column represents a mean of two experiments.

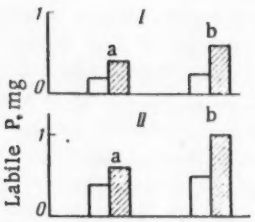

Fig. 2. Labile phosphorus content in the intestinal wall (a) and contents (b) of irradiated (I) and normal (II) rats. Unshaded columns, amino acid mixture without additions; shaded, amino acid mixtures with ATP, phosphate and Mg^{++}.

The nitrogen content of the intestinal wall fell considerably (from 21 mg/g in controls to ca. 12 mg/g), while the labile P content was not affected. There was no gross change in the assimilation of protein in the animals of this group.

The second experimental series dealt with the effect of radiation, adrenalectomy and cortisone excess on the absorption of amino acid mixtures and of histidine. It was found that in rats which have received 500-600 r irradiation the absorption of amino acids was somewhat inhibited and the stimulating effect of ATP, phosphate and Mg^{++} was somewhat lower than in control animals (Fig. 1).

While in control animals 25% of the added amino acids was absorbed, in irradiated animals the corresponding value was only 16%. The addition of ATP, phosphate and Mg^{++} to the mixture caused a twofold (48%) absorption in normal animals, while in irradiated rats the increase was only 21 — 35%. Histidine absorption was also inhibited in irradiated rats. In nonirradiated animals 20-26% of the histidine added alone was absorbed, while in the presence of ATP, phosphate and Mg^{++} absorption reached 33%. In irradiated animals, both in the case of histidine added alone and with the activators, absorption did not exceed 16% of the added material.

The labile phosphorus content of the intestinal wall and of the lumen contents fell in irradiated animals compared to controls. It is noteworthy that in irradiated rats (Cf. Fig. 2) the labile phosphorus content was higher when the added mixture contained ATP and phosphate, although even then the values did not reach those found in control animals.

The absorption of amino acid mixtures in adrenalectomized rats was studied 5-7 days after operation. The results are shown in Fig. 3. The columns in the upper row represent absorption in adrenalectomized animals, the lower row represents normal controls. While in normal animals absorption comprised 26% of the amino acids added alone and 48% of the amino acids added with ATP, phosphate and Mg^{++}, the corresponding values in adrenalectomized animals were 5-16 and 20%.

* Muscle extract with vasodilative properties - Publisher.

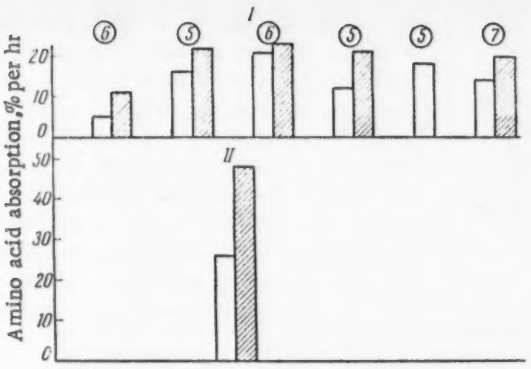

Fig. 3

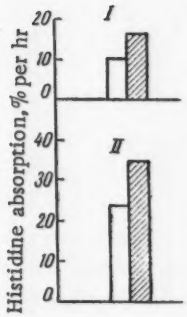

Fig. 4

Fig. 3. The absorption of amino acid mixtures in the gut of adrenalectomized (I) and of normal (II) rats. Numbers in circles denote days elapsed between adrenalectomy and experiment. Unshaded columns — 14% amino acid mixture without other additions; shaded columns — the same, with ATP, phosphate and Mg^{++} added.

Fig. 4. Histidine absorption from a mixture of amino acids administered to the intestine of adrenalectomized (I) and normal (II) rats. For key, see Fig. 3. Each column represents a mean of 10 experiments.

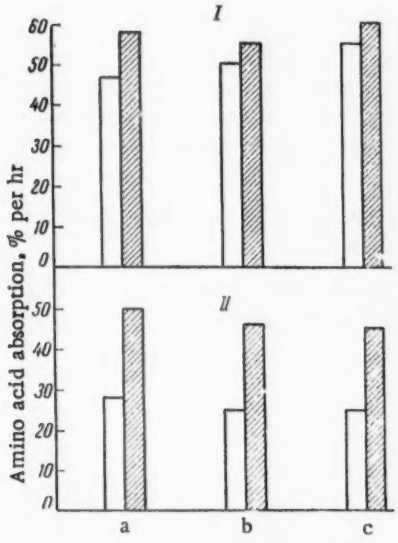

Fig. 5. Intestinal absorption of amino acid mixtures in cortisone-treated (I) and normal (II) rats. For key, see Figs. 1 and 3.

Absorption of histidine in the gut of the adrenalectomized rat was also found to be inhibited, both in the case of histidine present in amino acid mixtures and when 0.25 mole of histidine solution alone was introduced (Fig. 4). Of the histidine present in the amino acid mixture, 24% was absorbed in the normal rat intestine, while only 10% was absorbed in the adrenalectomized rat. In the adrenalectomized animal the addition of ATP, phosphate and Mg^{++} produced only an insignificant stimulation. When added alone, histidine was absorbed to the extent of 90% in one hour in the intact animal, while in the adrenalectomized animal absorption reached only 56% in the same time.

It follows from this part of the work that the adrenal hormones exerted some influence on the intestinal absorption of amino acid mixtures and of histidine alone. Adrenal cortical hormones, and particularly cortisone, influence, according to published data, both phosphorylation processes and the absorption of certain compounds. The present work dealt with the effect of excess cortisone on the absorption of amino acids.

The absorption of amino acids in the intestine of cortisone-treated rats was found to be considerably hogher than in normals (Fig. 5). In cortisone-treated animals the absorption reached 40-45% (control value: 25%) of the added material in one hour, in the absence of any additions, while in the presence of ATP phosphate and Mg^{++} 53-60% of the added mixture was absorbed (48% in untreated animals). Thus, the stimulating effect of cortisone was more pronounced in the absence of the additives.

Cortisone treatment also stimulated the intestinal absorption of histidine present in the amino acid mixtures (Fig. 6). The values found for histidine absorption in adrenalectomized (I) and normal (II) rat were 40 and 24%, respectively; when ATP, phosphate and Mg^{++} were added in the mixture, the corresponding values rose to 50 and 34% respectively.

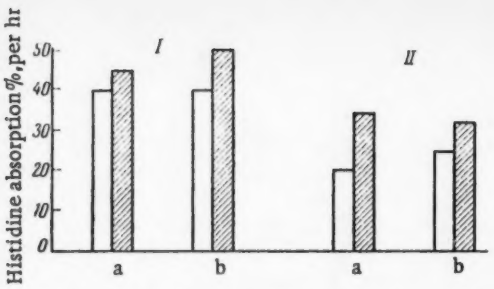

Fig. 6. Intestinal absorption of histidine present in amino acid mixtures in cortisone-treated (I) and in normal (II) rats. For key, see Figs. 1 and 3.

The concentration of labile phosphorus in the intestinal wall and contents of the lumen rose somewhat as a result of cortisone treatment; when the amino acid mixture alone was introduced, labile P concentration in the intestinal wall of cortisone-treated and normal animals was 0.6 mg and 0.4 mg, respectively, while with added ATP, phosphate and Mg^{++} the corresponding values were 0.75 and 0.6 mg. In the lumen contents the corresponding values (treated and normal) were 0.8 and 0.5 mg respectively with the mixture alone and 1.3 and 1.0 mg, respectively, with the additives present.

It follows from the present work that both x-irradiation and adrenalectomy inhibited the intestinal absorption of amino acids, while cortisone treatment stimulated this process.

It may be concluded from published work that x-irradiation as well as adrenalectomy interfere with oxidative phosphorylation. The present results show the similarity of the two treatment in inhibiting intestinal absorption, and the reserve effect by cortisone. The experimental results thus provide additional evidence for the hypothesis advanced by the authors, that in the intestine the absorption of amino acids is coupled with their phosphorylation.

SUMMARY

Protein assimilation, nitrogen balance and the nitrogen and labile phosphorus content of the intestinal wall of rats were virtually enaffected by x-irradiation in doses of 400 r.

When irradiation doses reached 500-600 r, the rate of amino acid absorption in the gut decreased. The stimulating effect of ATP, phosphate and Mg-ion added with the mixture was less pronounced in irradiated animals than in normal controls. Adrenalectomy was found to decrease considerably the rate of amino acid absorption in the intestine. On the other hand, the intestinal absorption of amino acid mixtures and of histidine in animals treated with excess cortisone was considerably higher than in controls.

Labile phosphorus concentration in the intestinal wall and in the lumen contents was also decreased in the irradiated animals compared to controls; it was also increased with cortisone treatment.

The decreased ability of adrenalectomized and x-irradiated rats to absorb amino acid mixtures and pure histidine from the intestine indicates that the intestinal absorption of amino acids is coupled with their phosphorylation.

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THE RATE OF RENEWAL OF PHOSPHORUS COMPOUNDS OF THE BRAIN IN DEEP HYPOTHERMIA

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Hypothermia in warm-blooded animals causes deep physiological changes in the state of the brain. Thus, in dogs when the body temperature is lowered to 28-30°, a cortical inhibition occurs which leads to cessation of conditioned reflex activity [1]. The rhythm of the electrophysiological activity of the brain at 33-31° is greatly slowed, and at 20° electrophysiological effects vanish almost entirely [1,2].

On the biochemical side, the state of the brain in hypothermia is chiefly characterized by a sharp fall in oxygen adsorption [3,4]. The inhibition of anaerobic glycolysis is shown to a much smaller extent [5]. In spite of the lower intensity of metabolism, the content of such energy compounds as ATP and phosphocreatin is not decreased [5,9]. Because of the great slowing of biochemical processes in hypothermia, even after 10 min stoppage of blood flow through the brain there still remain some phosphocreatin and about half the labile phosphorus of ATP, while at normal temperatures cutting off the blood supply for even 3 min lead to complete splitting of the phosphocreatin [5,8]. Hence we can understand the fact that in hypothermia animals can remain alive much longer when the work of the heart stops than can bodies at normal temperature.

Changes in the intensity of metabolism of various compounds are well characterized by the rate at which they take up labeled molecular groups. In our previous studies [10] on hypothermia we noted a sharp slowing of the uptake of a number of phosphorus compounds of the brain, and in the experiments of Vladimirov and Urinson [11] under the same conditions there was a sharp decrease in the uptake of labeled amino acids (methionine-S^{35} and tyrosine-C^{14}) in the brain proteins.

The first problem of the present investigation was to make a more detailed study than before [10] of the effect of hypothermia on the rate of renewal of a number of phosphorus-containing brain components (ATP, hexose monophosphates, phospholipids, phosphoproteins, and lipoproteins). The second problem was to answer the question of whether during hypothermia there was such damage to the enzyme systems concerned that it would appear in phosphorus metabolism after body temperature had returned to the normal level. Finally, we tested experimentally the reason for the fall in rate of phosphorus metabolism in narcotic sleep.

METHODS

The experiments were run on white rats weighing 180-200 g. About 15-20 min before cooling, the animals were injected with sodium amytal in an amount which produced narcotic sleep (7 mg/100 g body weight).

Deep hypothermia was produced by immersing the sleeping animals in water with ice. Under such cooling conditions, the rectal temperature fell to 20-18° after 10-15 min. Then the animals were injected subcutaneously with a solution of sodium phosphate with an activity of 0.5 μC per body weight. The rectal temperature (measured with a mercury thermometer or an electrothermometer) remained at a constant level within 1-2°.

As controls we used rat in a state of narcotic sleep which were periodically warmed by an electric lamp to maintain a body temperature of 35-37°. The animals were decapitated, the cerebral hemispheres were removed, dried rapidly with filter paper, weighed, and ground with 10 ml of ice cold 10% TCA.

After centrifuging, we determined inorganic phosphorus (by precipitation with magnesium mixture), labile phosphate (on the basis of ATP and ADP) and hexose phosphates in the supernatant liquid. In the tissue residues we analyzed for phospholipids, lipoproteins, and phosphoproteins by the method of Thannhauser and Schmidt, but with a number of changes, as we described previously [12].

Analysis for ATP and hexose phosphates was carried out by the method described in the work of Vladimirov and Rubel' [13] with the following modification. The mercury precipitate of ATP was dissolved in 2 ml of 1 N HCl and molybdenum reagent and eikonogen were added to the solution. After development of the color the samples were extracted with an equal volume of isobutanol. The alcohol layer which contained the inorganic phosphorus adsorbed on the mercury precipitate of ATP was removed and 2 ml of the aqueous residue was hydrolyzed for 10 min at 100°. The inorganic phosphorus thus formed by hydrolysis of ADP and ATP was precipitated according to Delory, the calcium-magnesium precipitate was dissolved in weak HCl and after the addition of molybdate and eikonogen the colored sample was again extracted with isobutanol.

The blue alcoholic solution served for colorimetric determination of phosphorus and for calculating the activity.

The supplementary extraction of inorganic phosphate from the ATP solution was necessary because in experiment without freezing the animal completely, only a small part (2-4 mg%) was kept undercomposed in the brain, and the content of inorganic phosphorus, on the contrary, rose considerably (to 30-35 mg %). In this connection, the mercury precipitate of ATP, ADP, and AMP could remove more than the usual amount of inorganic phosphorus. Also, the presence in the hydrolyzates of mercury precipitates of ATP of large amounts of AMP could introduce a further error due to removal of AMP in the calcium magnesium precipitate in the precipitation of inorganic phosphorus from the ATP hydrolyzate. The inclusion of AMP in the precipitate of inorganic phosphate would increase the radioactivity of the sample without changing the amount of inorganic phosphorus determined.

Phosphorus in most experiments was determined by the method of Fiske-Subbarow, but for analysis of samples with a very small phosphorus content we used the more sensitive method of Martin and Doty in the modification of Baranov [14]. Measurement of the radioactivity was carried out on a type B apparatus with a tube or end counter.

RESULTS

Effect of Hypothermia on Phosphorus Metabolism in the Brain

The data which we obtained indicate that the specific activity (SA) of inorganic phosphorus of rat blood in a state of hypothermia is definitely higher than in the control animals (Table 1). This is evidently related to the slower transfer of radioactive phosphorus from the blood into all the organs and tissues in such animals.

The contamination of brain tissues with blood with high activity can introduce great errors in the determination of the specific activity of the inorganic phosphorus of brain tissue.

Attempts to correct for blood contamination by determining the blood left in the brain did not give satisfactory results in our conditions since such corrections were very large and not very accurate. Therefore we preferred to remove the contaminating blood by perfusion. For this purpose, the thorax of the animal was quickly opened at the end of the experiment, the aorta was located, the descending branch was pinched off and the jugular vein was cut on both sides. We injected through the aorta (at the level of the arch) 30-40 ml of physiological saline cooled to 2-3° alternately into the left and right carotid artery, using a blunt needle from a syringe.

The perfusate from the brain had a light yellow color. The iron content in it was 13-15 $\mu g/g$ which, as special experiments showed, corresponded to full freedom of the brain tissues from blood [15]. The need for the use of perfusion in the experiments on hypothermia is confirmed by the data given in Table 2.

We showed that while in the control animals the value for SA of inorganic phosphorus of perfused and unperfused brain was of about the same order, in rats in hypothermia the value of SA of brain inorganic phosphorus with perfusion was almost three times lower than in experiments where perfusion was not used.

TABLE 1

Effect of Hypothermia on SA of Blood Inorganic Phosphorus SA in counts/min · mg P (P^{32} injected in amount of 0.5 μ curie/g body weight; duration of experiment 2 hours)

Control, temperature 36-37°	Hypothermia, temperature 18-22°
1 338 000	2 268 000
1 175 000	3 409 000
1 592 000	3 120 700
1 461 900	3 624 200
1 433 300	3 233 300

TABLE 2

Effect of Removal of Blood by Perfusion on the Values for Inorganic Phosphorus in Brain Tissue (SA, in counts / min · mg P; P^{32} injected in the amount of 0.5 μ curie/g body weight; duration of experiment 2 hours)

Control, 36-37° C		Hypothermia, 18-22°C	
No perfusion	With perfusion	No perfusion	With perfusion
13 400	14 000	40 000	11 330
19 900	23 170	54 200	12 250
14 700	16 310	28 200	14 570
13 610	12 500	36 500	12 400
16 660	16 700	20 430	13 940
21 260	20 000	21 670	6 350
18 250	19 200	21 670	4 600

When the animals were killed by freezing them whole, the differences in activity of brain inorganic phosphorus in the experiments with and without perfusion were obviously still greater; also, the individual variation from experiment to experiment could increase. Therefore we did not freeze the animals whole, although this method is the best for fullest preservation of the labile phosphorus compounds.

For characterization of the intensity of metabolism of inorganic phosphorus compounds we must calculate the relative specific activity (RSA) which is the ratio of SA of the phosphorus of the studied compounds to the SA of inorganic phosphorus and which is usually expressed in percent. In some of the work we used the SA of the whole acid soluble fraction of the SA of the inorganic phosphorus.

As the results given in Table 3 show, it is important for the true indication of the effect of hypothermia on the rate of renewal of this phosphorus fraction which SA we take for comparison. The relation of specific activity of P in the studied compound to the specific activity of all the acid soluble phosphorus gives higher values for the rate of metabolism since the acid soluble phosphorus contain some compounds which exchange their phosphorus slowly. Therefore such a calculation can be used only for obtaining orienting data.

ATP is a direct donor of phosphorus groups for the synthesis of many phosphorus compounds. Therefore we consider it more accurate to express the values for the renewal of the fraction studied by the relation of SA of their phosphorus to the SA of the phosphorus of the end group of ATP. At normal body temperature the renewal in ATP of both labile phosphorus groups occurs quickly and therefore the SA of labile ATP phosphorus, including also the labile phosphorus of ADP, is practically identical with the SA of the inorganic phosphorus (Tables 3 and 4).

In hypothermia the ratio is different. The SA of labile ATP is much lower than the SA of inorganic phosphorus (Table 4). Evidently this is first of all because the second phosphate group even after two hours does not have time for full restoration from inorganic phosphate.

Since in perfusion and the later treatment of the brain tissue a considerable part of the labile phosphorus is split, it is of interest to see whether in the extracts studied there is any actual portion of ATP. For this purpose we ran experiments with paper chromatographic separation of the nucleotides present in the mercury precipitate after precipitation from the TCA filtrate.

The mercury precipitate of ATP was dissolved in a small amount of weak HCl and after decomposition by a stream of H_2S, the clear solution was placed on the chromatogram. We used single phase chromatography with successive use of acid (10 hours) and alkaline (85-90 hours) mixtures of solvents at 20°. The first mixture was n-butanol-n-propanol-acetone-formic acid 30% TCA (40:20:25:25:15) (Gerlach et al. [16]); the second mixture was n-propanol-water-ammonia (60:35:5) (Venkstern and Baev [17]).

Inspection of the chromatogram with the ultrachemiscope showed the presence of five sharply separated spot which contained phosphorus and had an extinction at wave length 254 m μ. Three of these, in agreement with the positions of standards, corresponded to ATP, ADP and AMP. so that according to the phosphorus content and its radioactivity, about a third of the labile phosphate of the mercury precipitate belongs to ATP, and the remainder to ADP and other compounds. In connection with the predominance in the mercury precipitate of the second phosphate group of ATP and ADP, and the apparent incomplete renewal of this group, the specific activity

TABLE 3

Ratio of SA of Different Phosphorus Compounds from Brain Tissue depending on Conditions of Calculation (P^{32} injected in amount of 0.5 μcurie/g body weight)

Index	Control 36-37° C		Hypothermia, 18-20°C	
	expt. 1	expt. 2	expt. 3	expt. 4
SA:				
P_{as}	12 100	13 200	2 660	—
P_i	16 700	20 000	6 350	12 400
P_{ATP}	19 350	22 330	3 450	8 130
Hexose phosphates				
SA	6 230	6 560	691	2 550
RSA with respect to:				
P_{as}	51	50	26	—
P_i	37	33	11	18
P_{ATP}	32	29	20	28
Phosphoproteins				
SA	5 300	6 800	747	1 570
RSA with respect to:				
P_{as}	44	51	28	—
P_i	32	34	12	13
P_{ATP}	27	30	22	19
Lipoproteins				
SA	3 700	3 730	190	473
RSA with respect to:				
P_{as}	31	28	1,0	—
P_i	22	19	3,0	3,8
P_{ATP}	19	17	5,5	5,8
Phospholipids				
SA	220	229	base	21
RSA with respect to:				
P_{as}	1,8	1,7	0,0	—
P_i	1,3	1,2	0,0	0,2
P_{ATP}	1,1	1,0	0,0	0,3

Designations: P_{as}) Phosphorus in the acid soluble fraction; P_i inorganic phosphorus; P_{ATP}) labile ATP phosphorus.

of the mercury precipitate phosphorus in hypothermia was lower, and the RSA with respect to the SA of this phosphate was higher, than calculated in respect to the SA of inorganic phosphorus. Therefore, in considering the data of the main series of experiments (Table 4) we give the values of RSA calculated both in respect to inorganic phosphorus and to labile ATP phosphorus. If the first value is evidently slightly high, then the second, on the contrary, will be low. The true value of the RSA with respect to the activity of the end phosphate group of ATP should have an intermediate value.

It is clear from the foregoing data that hypothermia markedly lowers the rate of renewal of all the phosphorus compounds of brain tissue which we studied.

The greatest interference with revewal is found for compounds which have plastic significance; thus the RSA of the phospholipids falls by 80%, of the lipoproteins by 63%. The least interference with renewal of phosphorus is found in the phosphoproteins.

TABLE 4

Effect of Deep Hypothermia on RSA of Phosphorus Compounds of Brain Tissue (P^{32} injected in amount of 0.5 μcurie / g body weight; duration of experiment 2 hours; we give average values of RSA from 7 experiments; in parenthesis we give limits of variation)

Experimental conditions	RSA of phosphoproteins		RSA of hexose phosphates	
	to inorganic	to ATP phosphorus	to inorganic	to ATP phosphorus
Control, body 36-37° C	37 (32—49)	36 (27—46)	31 (27—37)	30 (26—34)
Hypothermia, doby 18-20° C	19 (12—28)	23 (19 - 30)	17 (11—23)	22 (13—28)
Percent decrease of RSA	48,6	36,1	45,1	26,6

Experimental conditions	RSA of lipoproteins		RSA of phospholipids	
	to inorganic	to ATP phosphorus	to inorganic	to ATP phosphorus
Control, body 36-37° C	17 (14—22)	17 (14—19)	1,09 (1,0—1,3)	1,03 (0,85—1,22)
Hypothermia, body 18-20° C	4,9 (3,0--6,3)	6,2 (4,5-7,6)	0,17 (0,0—0,3)	0,20 (0,0—0,5)
Percent decrease of RSA	71,1	63,5	84,4	80,5

TABLE 5

Rate of Renewal of Rat Brain Tissue Phosphorus Compounds after Remaining in a State of Hypothermia (18-20° for 1 hour) (study made 2 hours after injection of P^{32} in an amount of 0.5 μcurie/ g body weight; average values of RSA; in parenthesis, limits of variation)

Experimental conditions	Number of expt.	RSA with respect to inorganic phosphorus			
		hexose phosphate	phospho-proteins	lipo-proteins	phospho-lipids
Control, body temperature 36-37°	8	42 (31—52)	37 (34—46)	19,5 (16—23)	1,3 (1,1—1,5)
Injection of P^{32} 30 min after reaching normal body temperature	8	44 (31—52)	47 (29—58)	19,0 (15—22)	1,4 (0,9—1,7)
Injection of P^{32} 18 hours after reaching normal body temperature	2	44 (50; 38)	38 (38; 37,7)	21,0 (21; 20,9)	1,45 (1,5;1,4)

Occurrence of Renewal of Phosphate Compounds of Brain Tissue after Removal of the Hypothermia

There is great interest in the question of the degree of reversibility of the slowing of brain tissue metabolism caused by hypothermia. To answer this question, we kept rats in a state of hypothermia at body temperature 18-20° for an hour, and then for 15-20 min they were immersed in a bath with water temperature of 34-36°. When the rectal temperature had risen to 35-36°, they were injected with radioactive phosphate and followed until the body temperature remained at the normal level (36-37°). After 2 hours the brains of the animals were taken for investigation.

The results of these experiments (Table 5) show that even after a comparatively short period (15-20 min) our investigation indicates that phosphorus metabolism of brain tissue returns almost to normal. This gives us a basis for concluding that although deep hypothermia leads to considerable interference with the enzymatic systems

TABLE 6

Effect of Amytal Sleep on Rate of Renewal of Brain Tissue Phosphorus Compounds (P^{32} injected at 0.5 μcurie/g body weight; body temperature of animals 36-37°; experiments carried out without perfusion; average results given; in the parentheses are the limits of variation)

Time of expt., min	Number of expt.	RSA in respect to inorganic phosphorus			
		Amytal sleep		Relative rest	
		phospho-lipids	lipo-proteins	phospho-lipids	lipo-proteins
80	5	1,5 (0,8—1,3)	20 (17—27)	1,0 (0,8—1,2)	20 (18—24)
120	6	1,2 (0,75—1,3)	21 (16—26)	1,5 (1,3—1,8)	25 (23—31)
180	5	2,3 (1,7—2,7)	26 (23—29)	2,5 (2,0—2,8)	29 (27—32)

of brain phosphorus metabolism, it does not damage them. This is also confirmed by studies with longer periods after hypothermia. If after an hour of hypothermia and rapid subsequent warming the animal is injected with labeled phosphate, not immediately, but after 18 hours, brain phosphorus metabolism will not differ from that in the control animals. The observations on rats treated in this way show that in 3-4 hours after hypothermia they reach an alert state, willingly consume food, and in no way differ from normal rats.

These results agree well with the studies of Savchenko [5] who found a rapid recovery of energetic metabolism after hypothermia, and with the interesting observations of Andjus et al. [18] who by the use of special method of warming succeeded in restoring to life rats kept for one and a half hours at 0.5-1°. Such animals in a number of cases lived a long time and could produce offspring.

Thus, we can conclude that one to two hour keeping of animals in a state of hypothermia at 18-20° does not reproduce irreversible changes in the biochemical systems which share in phosphate metabolism of brain tissue.

Reasons for Decreased Metabolism of Phosphorus Compounds in Narcotic Sleep

In connection with the observed effect of hypothermia on the rate of renewal of brain tissue phosphorus compounds it is necessary to consider our earlier data [19] on the effect of narcotic sleep on the renewal of phosphorus compounds in the brain. In careful observations on animals under narcotic sleep we found that even during the first 20-30 min of sleep, the rectal temperature usually fell 2-3°. We did not take this temperature factor into account before. In order to determine its significance, we made a series of experiments in which we compared the RSA of rat brain phosphorus compounds in the wakeful state and in amytal sleep. In the sleeping animals the normal body temperature (36-37°) was maintained by periodic warming with electric lamps.

As the data in Table 6 show, in this case we cannot observe the inhibiting effect of narcotic sleep which we found earlier. Some tendency to decreasing the value of RSA in sleeping animals at normal body temperature exists, but it is not statistically significant and undoubtedly most of the effect which we found earlier was related to the change in temperature of the body.

SUMMARY

In deep hypothermia there is a great decrease in rate of transfer of radioactive phosphate from the blood to the brain, and a great difference is observed in the value of specific activity of inorganic phosphate of blood and brain. Therefore, in order to obtain a true value for the specific activity of brain inorganic phosphate in two-hour experiments on rats, it is necessary to free the brain tissues from the blood by perfusion with physiological saline while at normal body temperature this is not necessary. The rate of renewal of phosphorus compounds, especially phospholipids and lipoproteins in the brain, is sharply decreased in hypothermia. In the posthypothermal period at normal body temperature there is rapid and complete return to normal renewal rate. Thus, the

effect of hypothermia is inhibiting but not destructive to the enzymes or structural systems which take part in the phosphorus metabolism of the brain. It has been established that our earlier observation of a slowing of phosphorus metabolism in amytal sleep is basically due to the lowering of body temperature.

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TRANSFORMATIONS OF C^{14}-GLYCERINE ACID IN BEAN LEAVES

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Glyceric acid is one of the most important early intermediates in photosynthesis [1,2]. With short exposure of bean leaves in the presence of $C^{14}O_2$ there is a high level of uptake of labeled compound in glyceric acid; there is the suggestion of the possibility that this substance shares in the formation of amino acids, especially serine[2]; however, further transformations of glyceric acid as a product of photosynthesis or in general transformations in plant tissues have not been studied.

There is information in regard to transformation of labeled glyceric acid in animal tissues [3]. In these studies it was shown that the injection into rats of C^{14}-glyceric acid labeled on the carboxyl resulted in formation of serine labeled on the same atom. The formation of serine from glyceric acid, according to these results, goes through hydroxypyruvic acid formed from D-glyceric acid. It was also shown [3] that the formation of serine in the liver and kidney of different animals can occur in the presence of hydroxypyruvic acid and L-alanine by transamination as a result of the action of a specific transaminase. In this case the following reaction occurs: hydroxypyruvic acid + L-alanine → L-serine + pyruvic acid. There are also data [4] according to which in the leaves of parsley, lettuce, rye, carrots, and other plants, but not in seedlings of wheat, pea seeds, and beet roots, there exists an enzyme system which catalyzes the reduction of hydroxypyruvic acid with diphosphopyridinucleotide with the formation of D-glyceric acid. This reaction is reversible. Thus the possibility is not excluded that in plant tissues there is the system D-glyceric acid → hydroxypyruvic acid → serine. The possibility of such a path is shown indirectly by data obtained in the study of the dark fixation of $C^{14}O_2$ by the leaves of succulents [5,6], according to which serine is formed earlier than glycine, in contrast to experiments which showed the entrance of the α-atom of glycine into the β-position of an elaborated molecule of serine [7-9]. It has also been suggested [10] that there is another path of transformation of D-glyceric acid: D-glyceric acid, phosphorylated in the presence of ATP, changes to phosphoglyceric acid, then into phosphohydroxypyruvic acid and phosphoserine. The phosphorylated intermediary products, confirming the possibility of such transformation, have been isolated [11] from rat liver. It may be that the same transformations are fully possible in plant leaves in which glyceric acid can again be transformed into phosphoglyceric acid, like sugars which are constantly metabolized with phosphorus.

We have considered it desirable to carry out an investigation of the transformations of glyceric acid with a small amount of material in order to confirm the possibility of using micromethods for such an investigation as a matter of convenience. This problem depended in part on the ability to isolate labeled glyceric acid and to design experiments to study its transformations.

METHODS

For the experiments we took 10-to 12-day-old bean seedlings in the leaves of which there is a definite formation of glyceric acid in the early stages of photosynthesis [2]. Leaves were cut singly from the plants and each leaf was put separately for 30 sec in a chamber with labeled $C^{14}O_2$ in air [12]. After exposure, the leaf was first fixed for convenience in 96% alcohol cooled to −70°, and then boiled in water for 2 min; the solution was filtered and then treated with a cationite until further addition caused no lowering of pH. The filtered solution along with small portions of wash water was evaporated in a vacuum over NaOH at room temperature and the total radioactivity of the solution was determined. It was $1.13 \cdot 10^8$ disintegrations per min. For preparative isolation of labeled glyceric acid the dry residue obtained after evaporation in a vacuum was dissolved in water and deposited as a strip on a piece of chromatographic paper.

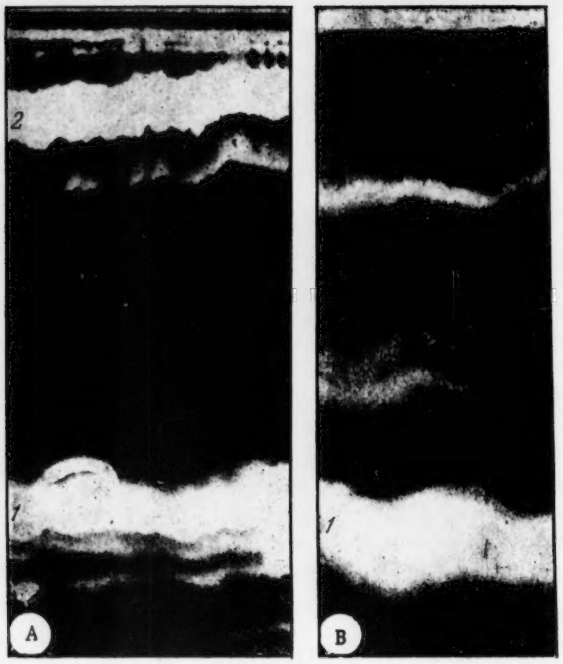

Fig. 1 . Radioautograph of the chromatogram. A) Leaf extract after exposure of the bean plant in $C^{14}O_2$. B) eluate of the zone of glyceric acid spot from chromatogram A. 1) Glyceric acid; 2) phosphoric esters of sugars .

Since in addition to glyceric acid there was formed in the bean leaf after 30 sec exposure a number of other radioactive substances [2] among which were phosphoglyceric acid, sugar diphosphates and monophosphates glucose, malic acid, aspartic acid, serine, and other compounds, it was necessary to choose a solvent whose use would permit the best separation of glyceric acid from the other substances on the chromatogram. Particular attention had to be paid to the separation of this acid from the labeled organic acids, especially malic acid, which with the solvents usually used in chromatograms were substances closely related to glyceric acid. Preliminary study of a number of solvents showed that the most suitable for the separation of glyceric acid from malic acid and other acids was one consisting of ethyl alcohol-ammonia-water (80:4:16). This solvent was also used for the preliminary separation of labeled glyceric acid. The resulting chromatogram was placed in a casette with an x-ray film XX. The results of the separation of the labeled compounds on the chromatogram with this solvent are shown in the radiophotograph (Fig. 1 A).

In this figure it is clear that with a 30 sec exposure of the bean plants the compounds among the phosphate esters are intensely radioactive; the strip of labeled glyceric acid is far separated from them. Since all the same there are compounds with the glyceric acid which have some, though it is true slight, radioactivity, the labeled glyceric acid was eluted with water and submitted to a second chromatographic purification on paper with the solvent butanol acetic -water (78:20:50). As a result of such purification we succeeded in separating completely the labeled glyceric acid from all the other labeled compounds (Fig. 1 B).

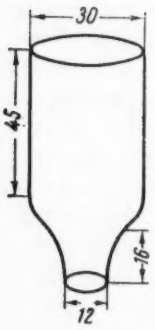

Fig. 2. Vessel for infiltration of small amounts of radioactive solution .

For further experiments one group of bean plants was illuminated with a 500 watt lamp for 40 min and another remained for the same period in the dark. A disc with diameter 11 mm was cut from the leaves and placed on the bottom of a special vessel (Fig. 2) which permitted infiltration with small amounts of solution. In the vessel we placed 1 ml of solution of labeled glyceric acid with a total radioactivity of $3 \cdot 10^6$ disintegrations per min, and carried out infiltration. After infiltration the leaf disc in the light experiments was illuminated for different periods and the disc in the dark experiments was kept for a corresponding period in the dark, after which the disc was fixed with boiling 80% alcohol. Extraction was repeated to full removal of soluble substances. The alcohol extract was evaporated in a vacuum at room temperature and the pigments and lipid were removed from it with ligroin.

The radioactivity of the separate fractions isolated from the leaves infiltrated with labeled glyceric acid is shown in Table 1. This shows that with longer exposure the radioactivity of the alcohol extract decreases and the activity increases in the insoluble material of the bean leaf discs. We note the fact that after two-hour exposure in the dark this activity is increased to 7.95 %.

In order to obtain an idea of the character of the radioactive substances dissolved in the alcohol extract we divided these substances into separate fractions. For this purpose, the dry residue of the alcoholic extract was dissolved in water and the resulting solution was deposited as a strip on a chromatogram. For the separation of the

TABLE 1

Distribution of Radioactive Products from Transformation of C^{14}-Glyceric Acid in Various Fractions of Bean Leaves (in percent of total radioactivity)

Experimental conditions	Exposure, min	In fraction soluble in 80% alcohol	In fraction insoluble in 80% alcohol	In fraction soluble in ligroin
Light	8	98,82	1,18	2,09
"	30	98,72	1,28	1,11
"	120	95,82	4,18	None
Dark	120	92,05	7,95	None

TABLE 2

Radioactivity of Different Derivatives of C^{14}-Glyceric Acid (in %) Found in the Alcohol Extract Fractions

Experimental conditions	Exposure, min	Organic acids	Amino acids	Substances obtained after separation of organic and amino acids
Light	8	29,9	65,8	4,3
"	30	44,7	52,8	2,5
"	120	52,6	30,4	17,0
Dark	120	26,2	65,1	8,7

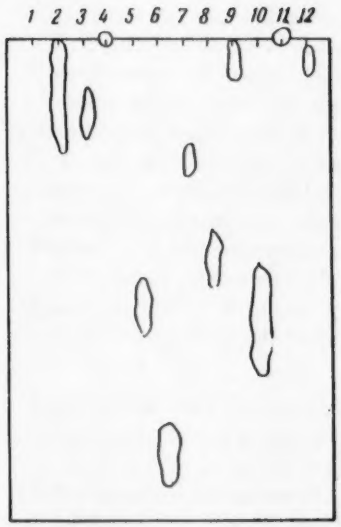

Fig. 3. Chromatogram of organic acids. 1) Oxalic acid; 2) citric; 3) malic; 4) phosphoglyceric; 5) succinic; 6) fumaric; 7) glycolic ; 8) malonic; 9) tartaric; 10) aconitic; 11) glucuronic; 12) glyceric. Solvent : ether-formic acid-water (14:0.2:1.8) by volume.

organic acids from the other compounds we passed a solvent: ether-formic acid-water (14:0.2:1.8).

A special test showed that some organic acids (tartaric, glyceric) are poorly soluble in this solvent and do not migrate far from the starting point (Fig. 3). For this reason the experimental chromatograms were cut 0.5 cm from the starting line. The acids were eluted and the radioactivity of the organic acid fraction was determined. The solution was treated with cationite KU-1 which, according to the literature [13] fully adsorbs most of the distributed amino acids. After treatment by the cationite, we again determined the activity of the solution. The difference between the two determinations before and after treatment with KU-1 gave the activity of the amino acid fraction. The amino acids were eluted from the cationite with 6 N ammonia. In the solution after removal of the amino acids there remained chiefly sugars and phosphoorganic compounds whose radioactivity was slight.

The separation of the radioactive compounds of the alcohol extract into different fraction is shown in Table 2. The fractions obtained as a result of the separation of the alcohol extract consisted of organic acids, amino acids, sugars and phosphoorganic compounds and along with the total alcohol extract were submitted to chromatographic analysis.

For the two-phase chromatogram, we passed in the first direction phenol saturated with 0.1 N formic acid, and in the second direction, n-butanol-acetic acid-water (78:20:50). For the single-phase chromatogram we used the solvents: n-butyl alcohol-acetic acid-water (78:20:50), ethyl alcohol-n-butyl alcohol-acetate ammonia buffer with pH 3.7 (4:1:2), and phenol-phosphate buffer with pH12 in the ratio 1:1. The chromatogram was shown with x-ray film and an exposure of several months.

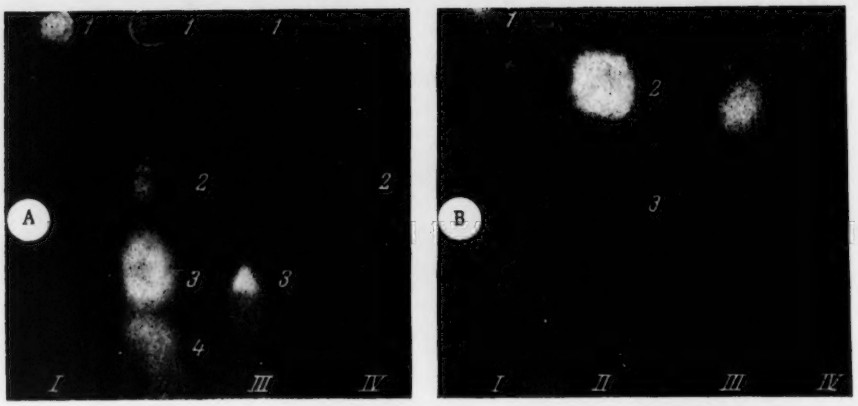

Fig. 4 . Radioautograph of chromatogram of alcoholic extract fraction from leaf discs infiltrated with C^{14}-glyceric acid. I) Light 8 min; II) 30 min; III) 120 min; IV) dark 120 min. A) Organic acids; 1) acids which remain at the point of application; 2) acid with Rf 0.09; 3) with Rf 0.17; 4) with Rf 0.20; B) amino acids ; 1) aspartic acid; 2) serine; 3) alanine.

On the chromatograms with solutions from the experiments on transformation of labeled glyceric acid in bean leaves in the light for 8 min , we observed three radioactive compounds: aspartic acid, an organic acid with high radioactivity, and which remained at the starting point in separation of the organic acid fraction by the solvent n-butyl alcohol-acetic acid-water, and glyceric acid with had not been transformed into other compounds. With exposures in the light for 30 min, labeled glyceric acid underwent further changes. We observed on the chromatogram seven radioactive compounds of which five were in the organic acid fraction. One of these, just as in the 8 min exposure sample, remained at the starting point, another was residual glyceric acid, and the remaining three acids could not be identified since the radioactive spots did not agree with any of the labeled substances used: glyceric , α-ketoglutaric, citric, malic, glycolic, fumaric, and aconitic acids. The spots of all these substances were distributed below the radioactive spots. The R_f of these unidentified acids in the solvent n-butyl alcohol-acetic acid-water were 0.09, 0.17, and 0.20. Besides the organic acids, with this exposure, we found strongly labeled serine and weakly labeled alanine. With a 2 hour exposure in the light we found five radioactive compounds of which three were in the organic acid fraction (one at the starting point, another with Rf 0.17, a third, a trace of glyceric acid), and two, serine and alanine, in the amino acid fraction. In two-hour dark exposure because of the weak radioactivity of the alcohol extract we could not identify separate compounds. We observed only the uptake of a labeled atom in an organic acid with Rf 0.09 and in the acid which remained at the starting point in the organic acid fraction. Since these substances were more apparent in the experiments in light, with short exposure, we believe that the transformations of glyceric acid in the dark are analogous to those in the light but occur more slowly. The radioautographs which illustrate the transformations of labeled glyceric acid in the compounds of the organic and amino acid fractions are shown in Fig. 4.

Fig. 4 A shows that in the organic acid fraction an organic acid remains at the starting point and its radioactivity with increased exposure gradually decreases. As to the amino acid fraction, Fig. 4 B shows that with 30 min exposure there is strong transformation of labeled glyceric acid into serine. The formation of alanine at this exposure occurs slowly. With longer exposure (2 hours) there is considerably less serine but alanine is formed intensively. For paper chromatographic separation of the fraction of sugars and phosphoorganic compounds we used 85% ethyl alcohol in which phosphate esters of the sugars are poorly soluble and the sugars move sufficiently far. The resulting radioactive spot is only at the starting point; it is weak because of the slight radioactivity of the fraction (Table 2). We believe that sugars are absent from this fraction, and it is possible that it contains phosphate esters of the sugars.

SUMMARY

Using paper chromatography we have isolated C^{14}-glyceric acid from bean leaves. Infiltration of labeled glyceric acid into bean leaves in the light and in the dark results in its intensive transformation chiefly toward formation of organic and amino acids.

The chief portion of the radioactive carbon of the glyceric acid in the light and dark experiments was found in the alcohol insoluble portion, and was evidently composed of high molecular weight substances, and a considerable part soluble in ligroin. Among the amino acids we identified chromatographically radioactive aspartic acid, serine, and alnine which appeared successively with increase in exposure from 8 to 120 min.

The formation of serine from labeled glyceric acid indicates that the path of transformation of this substance in the animal organism and the plant is similar.

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ASSIMILATION, BREAKDOWN AND NEW FORMATION OF AMINO ACIDS IN THE ORGANISM OF THE SILKWORM

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The problem of assimilation, new formation, and breakdown of amino acids in the silkworm has long attracted the attention of investigators. On the one hand, it is directly connected with the synthesis of those starting materials from which the silk protein is formed, and thus finally gives a basis for active intervention in this process. On the other, the problem is interesting in itself, both for its unusual nature and for the intensity of the amino acid metabolism which has been but little studied in insects.

Three different methods of investigation are combined in the solution of the question of the nature and extent of amino acid synthesis and breakdown in the silkworm: determination of the quantitative changes in amino acid content in the organism or tissues of the silkworm or in its food; study of the transformations of the amino acids in the silkworm under the action of enzymes; and study of the change of one amino acid into another during the life activities of the silkworm using radioactive indicators.

By quantitative determination of the amino acid content of the body and body tissues of mulberry and oak silkworm using balance experiments with and without feeding [1-9] it has been established that there is synthesis of alanine, phenylalanine, tyrosine, and tryptophan in the first and alanine and glutamic acid in the second. There has also been shown the deamination of alanine [5], deamination and oxidative decarboxylation of arginine [10] and extensive involvement of glutamine in metabolism; in the oak silkworm this can be regarded as an amino group depot [6].

The study of the enzymatic reactions of amino acid breakdown and synthesis leads to the conclusion that there are various paths both for destruction and building up of amino acids in the silkworm [11-15]. Breakdown occurs by deamination in the transamination reaction, by β-decarboxylation, or by dehydrogenation of the amino acid. Synthesis occurs chiefly by transamination of the amino acids with α-ketoglutaric, oxaloacetic, pyruvic, and glyoxylic acids, and also by direct reaction of β-decarboxylation of monoaminodicarboxylic acids. In a series of studies by Yamafuji and co-workers [16-22] the existence has been shown of a new type of amino acid synthesis in the organism of the mulberry silkworm from nitrogen-containing inorganic salts through the oximes with participation of nitratases, oximases, and transoximases. Glycine, alanine, aspartic and glutamic acids are synthesized in the silkworm.

Ideas on the transformations of amino acids in silkworms have been greatly broadened by studies of these reactions with radioactive indicators. Fakuda [23,24], using 2-C^{14}-phenylalanine showed its transformation into tyrosine. The same result, but with 1-C^{14}-phenylalanine, was obtained by Florkin and co-workers [25]. The list of amino acids synthesized by the mulberry silkworm has been considerably widened by the work of Sisakyan and Kuvaeva [26,27] who found the occurrence in the cavity fluid of the pupa during metamorphosis of the synthesis of glutamic and aspartic acids, alanine, serine, glycine, tyrosine, histidine, threonine, and arginine. The results obtained by the above three groups, which sometimes overlap each other, permit the drawing of conclusions as to the nature of the processes which chiefly determine the direction of transformation of amino acids in the silkworm. It is quite clear that the "highway" of metabolism of amino acids in the silkworm is the transamination reaction.

However, the quantitative side of amino acid transformations in silkworms remains to be worked out. In the

overwhelming majority of cases some transamination reaction has been chosen qualitatively as a reaction which is possible and occurs in model experiments with tissues and tissue extracts. There are only rare and fragmentary data as to the amounts of some amino acids which are synthesized in the life activities of the silkworm. There is no quantitative information at all on amino acid breakdown. Most often these and other results concern the larva stage V and the pupa and are lacking for other stages in the development of the silkworm.

We have chosen the problem of explaining what amino acids and how much of them are formed and broken down in the different stages of silkworm development.

METHODS

For a solution of our problem we carried out quantitative determination of the amino acids in the body, food, and excrement of silkworm at different stages in their development.* The caterpillars, beginning with age I, were fed under natural conditions on oak bushes. The food (oak leaves) was prepared from the same bushes by soaking followed by drying The excrement of the caterpillars was collected over several days of the experimental part of each age , sprinkled with a dilute solution of hydrochloric acid, and dried in air. The silkworm caterpillars and pupae were fixed by immersing in ten times the amount (by weight) of boiling ethyl alcohol. Average samples of material were taken in all cases.

For the preparation of the material for analysis the oak leaves were ground in an "Excelsior" type mill, the excrement was ground in a coffee mill, and preparations of oak silkworm caterpillars and pupae after removal of the alcohol in a vacuum and drying over sulfuric acid were ground in a mortar. The ground-up oak leaves and the oak silkworm caterpillar and pupa preparations were treated with dry ether in a Soxhlet apparatus.

In the defatted caterpillar and pupa preparations and oak leaves and also in the excrement which has not been treated with ether, we determined the sum of the free and bound amino acids. For this purpose a 0.02 to 0.04 g sample of the material weighed on a microanalytical balance with an accuracy to 10^{-5} g was hydrolyzed in a sealed glass ampule. with 10-15 ml of 20 % hydrochloric acid (distilled in glass) for 24 hours at 105-110° . Hydrolysis was carried out under conditions which assured continuous contact of the hydrolyzed material with hydrochloric acid and guaranteed a higher degree of agreement of results than the usual method of acid hydrolysis of protein [28]. The hydrochloric acid was distilled in a vacuum at 40° and the dry residue was dissolved in distilled water acidified with HCl. We used about 1 ml of liquid for solution, calculating the added amount by weight (on an anlytical balance with an accuracy of 10^{-4} g). The resulting solution was transferred by a filter pipet to a small tube and dried in a desiccator over sodium hydroxide. The sample was kept in this condition in a refrigerator until analysis.

The quantitative determination of the amino acids in the sample was carried out by paper chromatography as described previously [29].

Besides the amino acid composition we determined the total nitrogen content (by the Kjeldahl method) in the defatted and whole samples, and the silica content in whole samples [30] in order to carry out the corresponding calculations on assimilation and balance of nitrogen compounds. All calculations were based on absolute dry weight of samples. The amino acid content in all cases was calculated on dry weight of substance of the non-defatted preparation (with respect to nitrogen in whole and defatted samples).

RESULTS

In some of the analyses we calculated the amount of amino acid in the food of the oak silkworm (oak leaves) and in the excrement of caterpillars of age V. We also determined the quantitative amount of amino acids in the body of the oak silkworm at different stages of its development : at the begining of ages I and V, before transformation to the cocoon and pupa. These results permits us to explain some of the problem of assimilation, synthesis and breakdown of amino acids in the silkworm.

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* The material for analysis was obtained from the experimental station of the Zoological Institute , Academy of Sciences, USSR where summer and autumn feeding of oak silkworms was carried out. For supplying the material and for aid in the work we express thanks to the Director of the Division of Ecology of the Institute, Professor N.N. Sinitskii .

TABLE 1

Assimilation of Amino Acids by Age V Caterpillars of the Oak Silkworm

Name of amino acid	Amino acids obtained by the silkworm from the food per 100 g excrement, g	Amino acid content in 100 g dry excrement of the silkworm, g	Assimilation of amino acids, %
Lysine	0,7734	0,2713	64,92
Tyrosine	0,4673	0,1695	63,73
Glutamic acid	1,1056	0,4065	63,23
Valine	0,7042	0,2608	62,11
Proline	0,8662	0,3288	62,04
Alanine	0,6419	0,2461	61,66
Arginine	0,6283	0,2437	61,21
Phenylalanine	0,9747	0,3845	60,55
Serine	0,5764	0,2302	60,06
Leucine and isoleucine	1,4306	0,5881	58,89
Threonine	0,6281	0,2602	58,57
Histidine	0,4344	0,1872	56,91
Cystine and cysteine	0,1374	0,0597	56,55
Glycine	0,5925	0,2722	54,06
Aspartic acid	0,9522	0,4409	53,70

Assimilation of Amino Acids of the Food

Calculation of the assimilation of oak silkworm caterpillars of age V for each separate amino acid was carried out by the principle formulated by Arsen'ev [31] in a study ofsilkworm assimilation of nitrogenous substances. We multiplied the value of the content of amino acids in 100 g of dry leaves by the factor 1.203 (ratio of the amount of silica in the escrment to amount of silica in the food) and obtained the amount of amino acids which entered the silkworm in the food per 100 g of excrement. Knowing the content of amino acids in the excrement, we calculated the percent assimilation of the amino acids. The data for age V caterpillars are given in Table 1. The assimilation of food amino acids (Table 1) occurs selectively by the oak silkworm caterpillar with a range of 11%. Lysine, tyrosine, and glutamic acid are most completely assimilated; the percent assimilation of histidine, cystine, and glycine is less. In the mulberry silkworm the range in degree of assimilation of the separate amino acids reaches 17% and the list of well and poorly assimilated amino acids almost agrees with that for the oak silkworm [32].

The reason for the selective assimilation of the separate amino acids by the silkworm is not clear. There is no correlation between the order of assimilation of the amino acids and their use as essential or nonessential amino acids, or to the amount of amino acids secreted with the silk protein or synthesized on a considerable scale in the life processes. Furthermore, for some of the amino acids mentioned above the comparison leads to very unusual results. For example, glycine is secreted in large amount with the silk protein , yet the assimilation is less than for the great majority of the amino acids; although tyrosine is synthesized in the caterpillar, it is completely assimilated from the food, etc. It is possible that in the assimilation of amino acids by the silkworm such factors are important as the unequal permeability of different amino acids through the intestinal walls (as has been observed for other animals); the effect of one amino acid on the absorption of another, the presence in the plant of a protein fraction which is difficult to hydrolyze; the relation of the digestion of the proteins and adsorption of the amino acids to the character of the substances which accompany them, etc. These questions require further experimental study.

Synthesis and Breakdown of Amino Acids in the Silkworm

In order to establish the extent of synthesis and breakdown of individual amino acids during the life activities of the silkworm we had to determine the amount of assimilation and deposition of each amino acid from the food of the caterpillar. The results of direct analysis of the amino acid content of the body of the silkworm at various stages of development permit us to estimate the deposition of each amino acid.

For the determination of the amount of assimilation of the amino acids we had to know the amount of food eaten by the silkworm caterpillar at any period. Since we could not carry out complete balance experiments , the knowledge of the amount of food taken in by the silkworm was obtained by calculation. Starting from the data of

TABLE 2

Length of Feeding Period at Each Age of the Oak Silkworm Caterpillar (calculated by comparing the amount of nitrogen deposited in the body, entering with the food, and excreted, by 100 individuals)

Age of caterpillar	Nitrogen assimilated from food (g) if period of feeding is taken		Difference between them, g	Deposit of N according to age, g*	Difference between amount of nitrogen deposited in the body and assimilated from the food**		Length of feeding period, days
	with excess	with defficiency			g	days	
I	5 days 0,0356	4 days 0,0285	0,0071	0,0302	0,0017	$\frac{0,0017}{0,0071}=0,24$	4,24
II	6 days 0,1642	5 days 0,1368	0,0374	0,1386	0,0018	$\frac{0,0018}{0,0374}=0,05$	5,05
III	6 days 0,4522	5 days 0,3767	0,0755	0,4319	0,0552	$\frac{0,0552}{0,0755}=0,73$	5,73
IV	14 days 2,4165	13 days 2,2439	0,1726	2,3209	0,0770	$\frac{0,0770}{0,1726}=0,45$	13,45
V	13 days 10,4516	12 days 9,6477	0,8039	10,3868	0,7391	$\frac{0,7391}{0,8039}=0,92$	12,92

* Value taken from experiment.

** In period of feeding with deficiency.

the amount of excrement eliminated on the average in a day at any given period and using the observed ratio of content of silica in the excrement and leaves for this period, we calculated the duration of the period of feeding for each stage and the amount of food eaten and given off in the excrement for the same period. The system of these calculations is clear from Table 2.

The good agreement of the calculated data with the actual length of feeding at each stage in the development of the caterpillars allows us to assume that the calculated values for the amount of food eaten and of excreta can be used to calculate the amount of amino acids obtained from the food, excreted, and deposited by the silkworm. These results are given in Table 3. It follows from the data of this table that at ages I-IV of the oak silkworm caterpillars, only one amino acid, cystine, can be synthesized. At age V in the caterpillar there is intensive synthesis of alanine and glycine; tyrosine and serine are synthesized in small amounts. The synthesis of all these amino acids rises sharply in the period of spinning the cocoon and the transformation of the caterpillar into the pupa, when, per 100 g of dry substance, several grams of alanine, glycine, and serine are formed. However, our experiments leave open the question of the presence of synthesis by the silkworm of aspartic acid (ages I-IV) and arginine (age V), since the figures given for the increase in these amino acids lie at the border of the accuracy of the method.

If the total amount of amino acids synthesized by the caterpillars at age V and in the period of metamorphosis of the caterpillars into pupae is taken as 100%, then the distribution of their synthesis at each stage will be as follows:

	Age V	Spinning cocoon and transformation of caterpillar into pupa
Glycine	24.87	75.13
Alanine	37.49	62.51
Serine	8.10	91.90
Tyrosine	27.35	72.64

Thus, spinning the cocoon and transformation of caterpillar into pupa is characterized by intensive reconstitution of the amino acids, as has already been observed by Sisakyan and Kuvaeva [27] for the period of metamorphosis of the pupa of the mulberry silkworm.

TABLE 3

Synthesis and Breakdown of Amino Acids by the Oak Silkworm (on absolute dry substance of 100 individuals)

Amino acid	Caterpillars of age I-IV				Caterpillars of age V				Spinning cocoon and transformation of caterpillar to pupa				Amino acid deposited, synth.(+) degr.(-), in toto, g
	amino acids deposited in body, g	amino acids assimilated from the food, g	amino acids synthesized(+) or broken down(-)		amino acids deposited in body, g	amino acids assimilated from food g	amino acids synthesized(+) or broken down(-)		amino acid content in body		amino a., synth. (+), degr. (-)		
			g	% of assimilated			g	% of assimilated	caterpil. before cocoon, g	pupa and silk	g	% amino a. before cocoon	
Glycine	0,6845	1,2319	—0,5467	—44,40	4,4441	2,9726	+1,4715	+49,50	5,1319	9,5769	+4,4450	+86,62	+5,3698
Alanine	0,8262	1,3754	—0,5492	—39,93	7,3458	3,6759	+3,6699	+99,84	8,1760	14,2962	+6,1202	+74,86	+9,2419
Valine	0,7365	1,2155	—0,4790	—39,41	2,1562	4,0625	—1,9063	—46,92	2,8950	2,1878	—0,7081	—24,45	—3,0934
Leucine and isoleucine	1,5818	2,5585	—0,9767	—38,17	4,5367	7,8226	—3,2859	—42,01	6,1259	4,5074	—1,5555	—25,39	—5,8181
Aspartic acid	1,0825	1,0444	+0,0381	+3,65	3,6127	4,7452	—1,1325	—33,87	4,6992	3,0486	—1,6506	—35,13	—2,7450
Glutamic acid	1,5688	2,1633	—0,5945	—27,48	4,4289	6,4926	—2,0637	—31,79	6,1046	4,7745	—1,3301	—21,79	—3,9883
Serine	0,7779	1,1381	—0,3602	—31,71	3,4365	3,2143	+0,2252	+7,01	4,2204	6,5234	+2,3030	+54,57	+2,1680
Threonine	0,7718	1,2992	—0,5274	—40,59	2,8145	3,4157	—0,6012	—17,60	3,5908	2,7948	—0,7960	—22,17	—1,9246
Cystine and cysteine	0,4120	0,2229	+0,1891	+84,84	0,6097	0,7211	—0,1114	—15,45	1,0243	0,7520	—0,2723	—26,58	—0,1946
Arginine	0,9204	1,0854	—0,1650	—15,20	3,7747	3,5718	+0,2029	+5,68	4,6995	3,6719	—1,0276	—21,87	—0,9897
Lysine	1,0343	1,3868	—0,3525	—25,42	2,9148	4,6639	—1,7491	—37,50	3,9537	2,8661	—1,0876	—27,51	—3,1892
Histidine	0,4668	0,9855	—0,5187	—52,63	1,5857	2,2950	—0,7093	—30,91	2,0550	1,9807	—0,0743	—3,62	—1,3023
Proline	1,1607	1,4288	—0,2681	—18,76	2,6957	4,9904	—2,2947	—45,98	3,8610	2,5697	—1,2913	—33,44	—3,8541
Phenylalanine	1,4149	1,5127	—0,0978	—6,47	4,5303	5,4804	—0,9501	—17,34	5,9701	4,0666	—1,8835	—31,65	—2,9314
Tyrosine	0,7102	0,7640	—0,0538	—7,04	2,9991	2,7621	+0,2370	+8,58	3,7124	4,3417	+0,6293	+16,95	+0,8125

Note. Calculation of synthesis and breakdown of amino acids in ages I to IV was carried out from the data for assimilation of amino acids in age V (Table 1).

How do the amino acids undergo general breakdown in the organism of the silkworm? As Table 3 shows, there is a group of amino acids (valine, leucine and isoleucine, glutamic acid, threonine, lysine, proline) which undergo 20-40% breakdown during any of the stages studied. Along with these, some amino acids which are strongly broken down in one stage are used very economically in another (aspartic acid, cystine, arginine, and histidine). It is possible that the peculiarities in the behavior of these amino acids is connected with some characteristic of their assimilation and metabolism in the silkworm, since the great reactivity of free aspartic acid, cystine, and histidine in the cavity liquid, for example, of the mulberry silkworm has already been described in the literature [33]. Finally, the degree of breakdown of a definite amino acid is directly related to the amount of synthesis of the amino acids formed from it. This is the fact in the case of phenylalanine and tyrosine.

If we compare the total amount of amino acid breakdown with the synthesis (30.0307 g and 17.5922 g respectively for 100 individuals in the whole period of development from larva to pupa) we must conclude that the source of nitrogen for forming amino acids again is the amino acids which break down in the silkworm organism. Such a conclusion agrees well with the earlier conclusions on the transamination reactions as the basis for the metabolism, and particularly for the synthesis of amino acids in the silkworm. On the other hand, our data allow us to make a number of more accurate conclusions as to the question of the real possibility of similar synthesis under the conditions of the living organism. Actually, the considerable increase in the amount deposited in the body over that obtained in the food for glycine, alanine, serine, tyrosine, and cystine contrasts with the absence of real synthesis of the other amino acids, in spite of the possibility of this observed in model experiments.

In this connection we should also have a clear understanding of nonessential and essential amino acids in insects, not relating it to the possibility or impossibility of synthesis of amino acids by the tissues of the organism under various conditions, but understanding by this the presence or absence of a true synthesis of amino acids under ordinary, unchanged experimental conditions of feeding.

As to the new formation of amino acids from the nitrogen of nitrates of the food, this can scarcely occur, especially since it has recently been observed that the tissues of the mulberry silkworm contain an inhibitor (perhaps uric acid) which blocks the reduction of nitrates into nitrites [34]. We can also assume that the ideas of many authors from model experiments of the ability of tissues of the silk worm to fix the nitrogen of ammonium salts with the formation of amino acids is of no serious significance for the synthesis of the latter in the organism of the developing caterpillar and pupa of the oak silkworm.

It has already long been known that there is a relation between utilization of nitrogen and the level of carbohydrates in the food of the silkworm and this can probably be explained from the viewpoint of the effect of carbohydrates on the synthesis of amino acids. Carbohydrates evidently form the corresponding ketoacids which are the construction materials for the synthesis of amino acids, especially clearly shown in the pupa stage [27,33,35,36]. The poorer the assortment of carbohydrates and ketoacids which arise from them, the lower will be the level of the transamination reaction and the greater will be the part of the nitrogen which splits from the amino acids and leaves the silkworm organism; therefore the lower will be the percent of amino acids and proteins utilized from the food.

SUMMARY

The assimilation of amino acids of the food of caterpillars of the oak silkworm is selective. The range in degree of assimilation of the separate amino acids reaches 10%. A correlation between degree of assimilation of amino acids and their application to the group of essential and nonessential amino acids which are quantitatively secreted with the silk amino acids and the amount of synthesis of amino acids in the organism of the silkworm has not been found.

As the silkworm develops, the predominating role of amino acid metabolism in it increases, reaching a maximum in the period of spinning the cocoon and the transformation of the caterpillar into the pupa.

The synthesis of glycine, alanine, serine, and tyrosine occurs in the silkworm on a large scale, reaching several grams of each amino acid per 100 individuals in the whole period of development from egg to pupa. The synthesis of serine leads but is equaled by the degree of synthesis of glycine, alanine, and tyrosine.

The particular path of amino acid metabolism in the silkworm is the transamination reaction. There is also a definite occurrence of interconversion of amino acids. Direct amination of ketoacids from the nitrogen of nitrates and ammonium salts is probably of limited occurrence.

Starting from the principle that estimation of the nonessentiality or essentiality of amino acids can be expressed on the basis of the presence or absence of true synthesis of amino acids in the organism of animals, we must add to the list of nonessential amino acids of the silkworm glycine, alanine, serine, tyrosine, and cystine.

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THE EFFECT OF TEMPERATURE ON THE NUCLEIC ACIDS OF B. LICHENIFORMUS

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Life at high temperatures is one of the most interesting phenomena of nature. Organisms which live at high temperatures (thermophils) are found among both animals and plants. Among the latter are microorganisms which live at 55° or higher [1]. A characteristic peculiarity of the thermophils is the rapid rate of multiplication with intensive metabolism and enzyme activity which results in hastened protein synthesis and accumulation of the biomass.

In protein synthesis the energy rich compounds and nuclei acids are of great value [2]. They can be considered as donors of high-energy, phosphorus-containing groups, and also as a number of coenzyme systems. Temperature, like the nucleic acids, is an energy factor which slows or hastens enzymatic processes.

Wellerson and Terrault [3] showed that at optimum growth temperature B. subtilis and B. stearothermophylicus contain in the greatest amount of RNA, determined by the presence of its pentose. Variations from the optimum, either at lower or higher temperatures decrease the content of nucleic acids in the bacterial cell. It is quite possible that unfavorable temperature conditions of life are reflected not only in the nucleic acids, but also in the proteins and other substances and in the total state of the bacteria. However, the authors do not give any data in this respect.

The purpose of our work is to study the effect of change of temperature from the optimum on the nuclei and content under normal conditions for the bacteria.

The object of the investigation was the strain B. licheniformus which has a high proteolytic activity, isolated by Brotskaya [4] from the residues in the waste water, fermenting at 50° in the methane vats of the Lublin water-purification station. This organism is widely distributed in nature and has great practical importance in the mineralization of organic residues in the soil and in waste waters. The growth temperature of the thermophilic variant of B.licheniformus varies from 55 to 58°. We obtained from it by repeated transfers over a long period at constantly decreasing temperatures cultures which grew at 44 and 30°. Thus, in this study we used the original thermophilic variant and also the adapted cultures, the mesophilic, growing at 30°, and the intermediate, growing at 44°.

EXPERIMENTAL PART

In order to obtain material, the microorganisms were raised on a meat-peptone broth (Hottinger) for 12 hours at 58°; 18 hours at 44°; and 24 hours at 30° [5]. The varying periods of growth corresponded to similar stages of development. The stages were first established according to morphological characteristics of the culture by counting the various bacterial forms (rods, rods with spores, and spores in a Thoma chamber), and we also determined the weight of the cells [5,6]. To obtain in cultures rich in spores we grew the bacteria for 60 hours at 58° and for 72 hours at 30°. Such cultures will be called "spore" cultures. At the end of growth the bacterial films were washed, first with tap water and then with distilled water to remove the culture liquid; the water was separated from the film by centrifuging at 1500-2000 rpm; the precipitate was lyophilized. In the bacterial mass we determined nucliec acid and total phosphorus according to Fiske-Subbarow [7], total protein and nonprotein nitrogen according to Barnstein [8], ashing was carried out according to Kjeldahl, ammonia was removed by a semimicromethod [8].

TABLE 1

Content of Phosphorus and Different Forms of Nitrogen in Cultures of B.licheniformus (in percent on dry weight)

Growth temperature of cultures, °C	Growth of cultures, hr	Phosphorus	Nitrogen		
			total	protein	nonprotein
Ordinary culture					
30	24	3,73	10,2	8,98	1,02
44	18	3,25	9,41	8,25	1,17
58	12	2,04	9,28	8,14	1,12
"Spore" culture					
30	72	7,1	10,03	8,8	1,20
58	60	5,35	8,47	7,41	1,02

Phosphorus and Nitrogen. The results of the determination of phosphorus and the various form of nitrogen in the bacterial mass of a culture of B.licheniformus are given in Table 1.

The results in the table show that the spore culture is richest in phosphorus. A sharp change in the content of nitrogen compounds (90% of which are high molecular weight substances) does not occur and the low percentage of nonprotein nitrogen indicates the absence of autolysis. This fact is especially important, since in these microorganisms there are highly active proteolytic enzymes.

Nucleic acids were determined in the bacterial mass according to a method based on the separation of RNA from DNA by alkaline hydrolysis of the material by the method of Schmidt and Thannhauser, as modified by a number of authors [9]. By this method it is possible to find the RNA content from the mononucleotides, and the DNA from the purine bases, using the method of chromatographic analysis and the determination of the optical density of the eluate in ultraviolet light. Though this method is difficult, it seems to us to be the most suitable for the analysis. Actually, the literature descriptions of the determination of total nitrogen of nucleic acids in bacteria by pentose and by nuclei phosphorus do not seem to be accurate enough because of the possible presence in the corresponding fraction of carbohydrates which do not belong to the nucleic acids, and also of contaminants of polyphosphates in the nucleic phosphate fraction. At the same time the preparative isolation of RNA and DNA in the pure form takes place with great loss of these compounds. The recently published method of Spirin [10] can be used only when the type of nucleic acid is known from other methods and is valid only in the absence of hydrolyzable substances which adsorb ultraviolet light in the same wave lenght as the nucleic acids.

For the analysis we took from 100 to 600 mg of bacterial mass which contained 11-12% water. All the numerical data are given for a weight of 300 mg. The bacterial was treated according to the following plan.

Plan of Isolation of Nucleic Acids and Their Components from Bacterial Preparations

Bacterial mass

↓ I

Removal of mononucleotides and low molecular weight compounds

↓ II

Partial separations of lipids

↓ III

Separation into DNA and RNA fractions

V ↓ Purification of DNA fraction

↓ VI

Hydrolysis of DNA to purine and pyrimidine bases

Mixture of purine and pyrimidine bases

↓ IV

Purification of mononucleotide fraction

Mixture of RNA mononucleotides

↓ VII

Separation of mixture of purine and pyrimidine bases and mononucleotides into separate components by paper chromatography and electrophoresis

↓ VIII

Quantitative measurement of separate mononucleotides, purine and pyrimidine bases by spectrophotometry in ultraviolet light

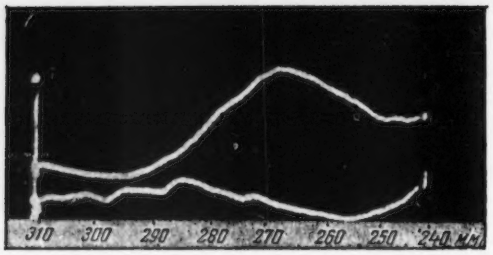

Fig. 1. Oscillogram of unknown spot. Ordinate) optical density; abscissa) wave length in mμ.

I. Separation of free low molecular weight mononucleotides whose presence could lead to incorrect determination of nucleic acids. These substances were removed by repeated digestion for 30-40 min with 2-3 ml of 0.2 N HCO_4* at 0 to 4°. Then the precipitate was separated from the solution by centrifuging at the same temperature. In all cases the separation of the precipitate from the solution at low temperature was carried out in a cooled centrifuge.

II. The precipitate was treated in the cold successively with alcohol, a mixture of alcohol and ether and with ether alone. By this treatment $HClO_4$ was removed, and also part of the lipids.

III. The dry residue was hydrolyzed in 4 ml of 0.5 N KOH at 37° for 18 hours. These conditions of hydrolysis were suitable for our culture (they were taken from the work of Spirin and Belozerskii [11]). At the end of the hydrolysis the alkaline solution contained mononucleotides from the split RNA, DNA, and some admixtures. DNA K ion, and a number of admixtures were precipitated in the cold with 50 % $HClO_4$ to a final concentration of acid acid in the mixture of 2-3%. The mixture was centrifuged at 0 - 3°. The residue contained DNA, K salts, proteins, and admixtures, and the acid solution contained RNA mononucleotides. The latter was combined with the wash water after washing the residual RNA mononucleotides from the precipitate, using 2-3% $NClO_4$.

IV. The mononucleotide solution was neutralized with KOH at 0-3°; the $KClO_4$ precipitate was separated by centrifuging at the same temperature, washed with ice water, and discarded. The wash and the rest of the solution was diluted to a volume of 15-25 ml. The resulting neutral solution of the nucleotide mixture served for chromatography and electrophoresis.

V. The DNA fraction was treated with alcohol and ether as described in part II. The fraction was put into a vacuum desiccator for complete drying. Then the DNA was removed from it in a weakly alkaline solution obtained by adding 0.7 ml of a saturated $NaHCO_3$ solution (4 ml of 10% NaCl). The whole mixture was heated for an hour on a boiling water bath, then cooled to 0° and centrifuged. In the precipitate, washed with 10% NaCl, there remained the admixtures including the proteins, and in the wash water and the solution was the DNA which was precipitated from solution by adding 2-3 volumes of 96% ethanol in the cold. For the best settling, the mixture stood for several hours at 0° and then was centrifuged and the resulting precipitate of DNA was finally dried in a vacuum desiccator. The yield of this fraction was 2-3% of the bacterial mass taken for analysis.

VI. The dry DNA fraction (10-25 mg) was transferred to an ampule which was filled with 72% $HClO_4$ (calculated at 0.2 ml of acid per 10 mg of sample). The ampule was sealed and hydrolysis was carried on for an hour on a boiling water bath. The hydrolyzate was diluted by adding an equal volume of water and was used in this form for the separation of the purine and pyrimidine bases of DNA by chromatography.

Chromatography. For chromatography. For chromatography and electrophoresis we used strips of washed Leningrad chromatographic paper No. 2 [6]. Chromatography was carried out by the descending method; the solution was passed in one direction.

Separation of DNA bases. For one chromatogram we took 50-100 μliters of dilute hydrolyzate. The conditions of separation have been described in previous work [6]. The order of bases progressively from the point of depositing the hydrolyzate was: guanine, adenine, cytosine, thymine.

Separations of RNA mononucleotides. The separation was carried out by chromatography and electrophoresis. For the chromatogram we took 100 to 600 μliters of mononucleotide solution. The separation was carried out in two systems of solvents. The composition of the first solvent was ethanol-butanol-1 M acetate ammonia buffer (4:2:1) [13]. The mixture separated into three spot in this solvent. In the first spot (from the starting point) we found uridylic and guanylic acids, in the second, cytidylic, and in the third, adenylic. After drying, the chroma-

* TCA, which has considerable optical density in the ultraviolet spectrum, was replaced by perchloric acid. The latter, according to Davidson and Smellie [12], adsorbs practically no ultraviolet light of the wave length used in our work.

TABLE 2

Calculation Coefficients for Mononucleotides

Mononucleotides	Wave length, mμ	K	Mononucleotides	Wave length, mμ	K
Adenylic acid	260	0,066	Cytidylic acid	270	0,146
Guanylic acid	250	0,094	Uridylic acid	260	0,103

togram was placed in the second solvent (prepared by our modification of the process of Magasanik [14]). To 25 ml of freshly distilled isobutyric acid we added 17 ml of twice distilled water and the mixture was allowed to stand in a separatory funnel at 0-3° in the refrigerator. After separation, the lower water layer was drawn off and to 31 ml of the upper layer was added 0.4 ml of concentrated ammonia to a pH of 3.5. The solvent prepared in this way was poured into a boat and the walls of a chamber lined with chromatographic paper were prayed with the same solution. After 10-18 hours the mixture had separated into mononucleotides distributed in the following order from the starting line: guanylic, cytidylic, uridylic, and adenylic acids.

Paper electrophoresis for the separation of the mixture into the four nucleotides was carried out in a special apparatus* in 0.05 M acetate buffer, pH 3.5, at 1-5°. For each analysis we took 50-100 μ liters of mononucleotide solution; the mononucleotides migrated to the anode, distributed in the following order from the starting point: cytidylic, adenylic, guanylic, and uridylic acids. For the mononucleotides from RNA obtained from cultures grown at 30 and 44° the separation took 6-7 hours at 870 v; for the thermophilic culture best results were obtained at 400 v. At the end of the electrophoresis the paper strip was dried at 80°. For the thermophilic culture we observed in the RNA nucleotide fraction a fifth spot, lying, like the nucleotides, on the anode side of the starting line. In Fig. 1 we give the adsorption curve of this spot taken directly from the electrophoretogram paper in the ultraviolet cytospectrophotometer [15]. This spot was not further studied.

Photography of the Chromatograms and Electrophoretograms

The photography was carried out in ultraviolet light [6]. In Fig. 2 we give the electrophoretogram of RNA mononucleotides from the mesophilic culture. Similar electrophoretograms were obtained for the thermophilic and intermediate cultures. The results of the distribution of bases in the DNA hydrolyzate repeat the picture which we found in the analysis of these compounds in the hydrolyzate of the bacterial mass, differing only in the absence of the uracil spot [5,6].

In Fig. 3 we give the chromatograms of RNA mononucleotides from thermophilic cultures. Fig. 3 A shows the separation of a mixture of mononucleotides in the first solvent; Fig. 2 B gives the picture after successive separations in the first and second solvents (in the second case all the chromatograms were taken without the neck since in the second solvent the substances descended considerably below it). The same type of chromatograms were obtained in the other cultures.

Depending on the RNA content in the cultures we took for the chromatograms varying volumes of hydrolyzate which corresponded to the taken sample of bacterial mass. Thus, for the mesophilic culture we needed an amount of hydrolyzate corresponding to a weight of 3.6 mg; for the thermophilic culture, 6 mg; for the intermediate and "spore" mesophilic cultures, 4 mg. In the "spore" thermophilic culture we increased the weight to 40 mg because of the very low concentration of mononucleotides.

Spectrophotometry of RNA Mononucleotides and DNA Bases

After inspection in the ultrachemiscope [16] and comparison with the photographs, the mononucleotide spots and the corresponding portions of the control chromatograms were marked with a pencil, cut out and ground up for best removal of the studied compounds.

The mononucleotides and the control parts were eluted from the paper with 5 ml of 1 M phosphate buffer

* The apparatus was constructed by Chief Enginer N. A. Chernov with the authors of this paper (experimental workshop, Moscow State University).

Fig. 2

Fig. 3

Fig. 2. Electrophoretogram of RNA mononucleotides of the mesophilic culture. 1) Cytidylic; 2) adenylic; 3) guanylic; 4) uridylic acids.

Fig. 3. Chromatograms of RNA mononucleotides of the thermophilic culture. A) Separation in solvent I; B) separation with solvents I and II. 1) Guanylic; 2) uridylic; 3) cytidylic; 4) adenylic acids.

TABLE 3

RNA Mononucleotide Composition of Bacteria Grown at Different Temperatures (in percent dry weight and in mole%; figures in parentheses)

Mononucleotides	Ordinary culture			"Spore" culture	
	30°	44°	58°	30°	58°
Guanylic acid	2,02(33,95)	0,82(31,45)	0,75(29,8)	0,49(28,62)	0,12(32,85)
Uridylic "	0,94(17,80)	0,47(17,81)	0,44(19,40)	0,51(23,69)	0,05(16,93)
Cytidylic "	1,25(23,85)	0,54(24,36)	0,43(20,8)	0,52(24,00)	0,09(23,27)
Adenylic "	1,37(24,40)	0,73(26,38)	0,71(30,0)	0,55(23,69)	0,08(26,95)

TABLE 4

Characteristics of RNA according to Chargaff

Temperature of growth of culture, °C	Native G+U/A+C	Type G+C/A+U	Purine / pyrimidine
Ordinary culture			
30	1,07	1,37	1,37
44	0,97	1,23	1,37
58	0,98	1,03	1,40
"Spore" culture			
30	1,09	1,11	1,10
58	0,99	1,28	1,40

Designations: G) guanylic; U) uridylic; A) adenylic; C) cytidylic acids.

with pH 3.0 for 12 hours [11]. The purine and pyrimidine bases were eluted with 5 ml of 0.1 N HCl at room temperature for 24 hours [6]. The concentrations of these compounds in the eluate was measured in a quartz cuvette 1 cm thick on an SF-4 spectrophotometer by the method of difference of extinction. In this case the true extinction equaled the difference between the maximum extinction and the extinction at 290 mμ [17].

The content of mononucleotides, purine, and pyrimidine bases in the bacterial mass is calculated from the formula

$$M = KV\Delta E/B$$

where M is the content of the compound mentioned, in μmole / g dry weight; K the calculation equal to the number of μmoles contained in 1 cm^3 with a thickness of layer of 1 cm which gives an extinction of 1 for the wave length used [17]; V the volume of the eluate in cm^3; B the weight of the bacterial mass in the spot taken for chromatography, in g.

The calculation coefficient for purine and pyrimidine bases is taken from the work of Spirin [13] which he calculated from the data of Beaven [18]. We also made a correction in regard to the removal of the purine and pyrimidine bases from the paper chromatogram. This work was carried out with pure preparations of these compounds [6]. The calculation coefficients for the mononucleotides are given in Table 2.

In the separation of the mononucleotides by the second solvent there occurs a partial breakdown of adenylic acid. Therefore the coefficient K which is given should be multiplied by the corresponding correction 1.1 [17].

TABLE 5

DNA Mononucleotide Composition of Bacteria Grown at Different Temperatures (in percent dry weight and in mole %; figures in parentheses)

Mononucleotides	Growth temperature of cultures		
	30	44	58
Guanylic acid	0,16(26,14)	0,11(26,07)	0,055(25,13)
Adenylic "	0,14(25,43)	0,098(25,18)	0,051(25,80)
Cytidylic "	0,12(24,56)	0,085(24,46)	0,045(25,12)
Thymidylic acid	0,11(23,87)	0,094(24,29)	0,048(23,95)

TABLE 6

Characteristics of DNA According to Chargaff

Growth temperature of cultures, °C	Native G+T / A+C	Type G+C / A+T	Purine / pyrimidine
30	1,00	1,05	1,06
44	1,01	1,02	1,05
58	0,96	1,02	1,04

Designations: G) guanylic; U) uridylic; A) adenylic; C) cytidylic acids; T) thymidylic acid.

TABLE 7*

Content of Protein, RNA, and DNA in Bacteria Grown at Different Temperatures (in percent dry weight)

Growth temperature, °C	Protein	RNA	DNA	Growth temperature, °C	Protein	RNA	DNA
Ordinary culture				"Spore" culture			
30	49,98	5,58	0,56				
44	48,60	2,56	0,40	30	52,5	2,27	0,23
58	48,44	2,23	0,21	58	45,69	0,34	0,23

* The protein in the bacteria was calculated by the difference between total amount of protein (found from protein nitrogen) and total amount of nucleic acids (DNA + RNA) calculated on dry weight. The quantity of nucleic acids was calculated by the sum of the nucleotides which composed them. With such a method of calculation, the percentage of error was 5% because lack of knowledge of the molecular weight of the nucleic acids prevented determination of the number of molecules of water liberated in combining the mononucleotides into nucleic acids. At the same time the weight of a molecule of water is slight compared to the molecular weight of the mononucleotides. Such a method of calculation seems more accurate to us than calculation of the total nucleic acid content by the nitrogen of the purine compounds. For comparison we calculated the RNA and DNA content in cultures of B. licheniformus according to the purine nitrogen of the mononucleotides and bases. We showed that in the mesophilic cultures the RNA was 5.80%, in the intermediate cultures, 2.71%, and in the thermophils, 2.61%. These results are high (for example, for the thermophilic culture the error was 11%) due to the excess in the RNA of purine compounds over pyrimidines. The results for DNA were about the same in calculations by both methods, since in DNA the ratio of purine to pyrimidine is nearly unity. Hence, calculations by the purine nitrogen can be used only in the case of equality or near equality to unity in the ratio of purine and pyrimidine compounds in the nucleic acids.

In our work we checked this correction with a pure preparation of adenylic acid. It was 1.08, and in the calculations was rounded off to 1.1.

In Tables 3 and 4 we give the characteristics of RNA and in Tables 5 and 6, those of DNA for different cultures of B. licheniformus.

It follows from the data of Tables 3 and 4 that in the RNA of all cultures the purine compounds predominate over the pyrimidine; thus RNA belongs to the G + C type in which the sum of guanidylic and cytidylic acids is

TABLE 8

Content of Protein, DNA, and RNA in One Bacterial Cell

Growth temperature, °C	Weight of cell in $1 \cdot 10^6$ μg	Protein in $1 \cdot 10^{-8}$ μg	RNA in $1 \cdot 10^{-3}$ μg	DNA in $1 \cdot 10^{-8}$ μg
30	1,84	91,96	10,20	2,03
44	1,57	76,30	4,02	0,63
58	1,26	60,03	2,81	0,26

greater than the sum of adenylic and uridylic acids. With increase in temperature the amount of RNA mononucleotides is lessened and also the ratio to each other changes so that there is a gradual approach of RNA to the type A + U in which the sum of adenylic and uridylic acids surpass the sum of guanylic and cytidylic acids.

From the data of Tables 5 and 6 it follows that with increasing temperature there is a decrease in total content of the nucleotides which make up DNA. The DNA of the cultures studied belongs to the loosely called G + C type in which the sum of desoxycytidylic and desoxyguanylic acids surpass considerably the sum of the desoxyadenylic and desoxythymidylic acids. The type of DNA at higher growth temperatures is practically unchanged.

The total amount of proteins and nucleic acids in the different cultures of B.licheniformis is given in Table 8 Table 8.

The data of Tables 7 and 8 show that increased temperature leads to decreased weight of the bacterial cell, lowered content in it of nucleic acids, and to relatively slight decrease in protein.

In the spore cultures the content of nucleic acids is lower than in the ordinary cultures of B. licheniformus. Such results agree with the information in the literature [19]. However, it must be said that these figures are orienting, since in the "spore" cultures the vegetative bacterial cells make up 28% of the total number of bacteria.

DISCUSSION OF RESULTS

The total content of nucleic acids is decreased as the life temperature of B. licheniformus is increased from 30 to 58°. In passing from the mesophilic to the intermediate cultures, that is, from 30 to 44°, the decrease in total amount of nucleic acids is greater (by about 50%) than with further rise in temperature from 44 to 58° . The same relation is found for ratio of content of ribonucleic acid to the proteins as such. Thus, for 30° it is 12.5, for 44°, 6.09, and for 58° ,5.04. It is possible that such an unequal shift for the transfer from 30 to 44° on the one hand and from 44 to 58° on the other is connected with a difference for these temperature intervals of the van't Hoff temperature coefficient. It is known that the hastening of biological processes and enzymatic reactions in the interval from low to medium temperatures is higher than for that from medium to high [20]. However, the absolute rate of reactions (within known limits) increases with rise in temperature.

Nucleic acids can be considered as donor of active groups of enzymes which take part in a number of reactions, including protein synthesis. Therefore the decreased nucleic acid content at higher life temperatures is evidently related to the increased number of turnovers of the enzyme molecule [21] which compensate for the smaller amount of these enzymes, and so indirectly compensate for nucleic acids needed in protein synthesis and other reactions. Actually, the percentage of proteins in ordinary cultures of B. licheniformus grown at different temperatures differs little in each case. This suggested explanation is not the only one possible and does not exclude other points of view on this question.

It has been shown in a number of studies that nucleic acids increase the stability of proteins toward high temperatures [22,23]. Synthetic nucleoproteins are more stable than simple proteins. According to the literature, nucleic acids are present in thermophilic cultures in amounts sufficient to prevent the coagulation of the proteins of these bacteria at 58°. At the same time, the content of nucleic acids in mesophil cultures [24] is higher than in thermophilic ones, but the mesophils cannot normally develop at high temperatures. Hence, to explain the thermophils only by the simple quantitative ratio between proteins and nucleic acids is not possible.

SUMMARY

In thermophils, intermediates, and mesophilic cultures of B. licheniformus grown at 58, 44, and 30°, there are chiefly high molecular weight organic nitrogen compounds.

Cultures which contain a larger number of spores are the richest in phosphorus.

Increased growth temperature of the microorganisms is accompanied by decreased weight of cells, lower total amount of nucleic acids, and change in the mononucleotide composition of RNA.

RNA and DNA belong to the ordinary types which are characteristic of microorganisms. In the RNA of all cultures the purine nucleotides predominate over the pyrimidine. This is especially clearly seen in the RNA of thermophilic cultures. Increased temperature does not affect the protein content of ordinary B. licheniformus cultures.

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THE EFFECT OF IONIZING RADIATION ON THE OXIDATIVE PROCESSES IN TEA AND TOBACCO LEAVES

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The fermentation of tea and tobacco is accompanied by important oxidative reactions of the tannins or the polyphenols and glycosides, respectively, which play a considerable role in the determination of the quality of the final product. In the ordinary fermentative process these changes are the result of the activity of a number of oxidative enzymes – polyphenol oxidase and others. In view of the important role of these oxidative processes in the fermentation of tea and tobacco, it was of interest to determine the effect on these reactions of ionizing radiations, inasmuch as irradiation of living cells is known to cause an accumulation in large amounts of the products of water radiolysis – the radicals OH, O_2H, and hydrogen peroxide, all of which are known to be strong oxidizing agents. It could be expected that the irradiation of leaves would cause direct oxidation of some substrates, as well as affect the general conditions under which the enzymatic oxidative processes took place; these phenomena would also be of interest in the study of the mechanism of action of ionizing radiation. The present work deals with this subject.

EXPERIMENTAL PART

Experiments with Tea Leaves

Methods: the material used throughout were tea leaves from a tea bush in the hothouse of the Institute. X-irradiation was carried out using a BF tube (tension 160 kv, current 7 ma, dose-rate 5,000-10,000 r/min). Occasionally a tube of the type TRTs-3a was used (tension 80 kv, current 200 ma, dose-rate 65,000 r/min). The dose-rate was continuously monitored by means of a standard methylene blue solution which was irradiated simultaneously with the material [1]. Electron irradiation was carried out with an electron-accelerator with a voltage 800 kv, current 1 ma, and dose-rate 5 millions r/min.*

The results of the irradiation of tea leaves were assesssed by the degree of tannin oxidation. The latter was measured as follows. Six to eight discs, approximately 1 cm in diameter each, were cut from the surface of the leaf and weighed on an analytical balance (the disc weights were 35-45 mg), some of the discs were irradiated while the remaining ones were retained as controls. Since one disc sufficed for analytical purposes, both experimental and control discs could be cut from one leaf. After irradiation both the treated and the normal discs were incubated in the thermostat and then fixed with steam. Each fixed disc was ground in a special apparatus (Fig. 1) and extracted with 2.5 ml of distilled water for 10-15 min on a boiling-water bath. The apparatus allowed both the homogenization and the extraction of the material. The lower ground-in pestle c was used for the grinding of the leaf, which was easily accomplished by hand-rotating the extended condenser handle of the hollow pestle. The apparatus was closed by means of the upper ground joint b and the rubber rings, and immersed into the boiling bath. The reflux condenser ensured the constant volume of the extract. Regular boiling was supported within the pestle by an opening a in the pestle wall. At the end of the extraction period an additional 2.5 ml of water was

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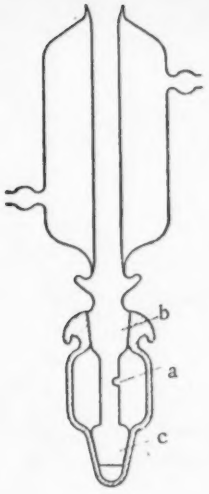

Fig. 1. An apparatus for the homogenization and extraction of small quantities of plant material. a) Opening in the combined pestle-condenser; b) upper ground joint; c) lower ground joint.

added through the condenser in order to wash down all fluid from its wall. The apparatus was carefully dried at the ground joint, opened, and the suspension was filtered through a glass filter, the filtrate being used for analysis. All calculations were made on the basis of the total solution, the volume of which was 5 ml. Tannin was estimated according to Levental' [2] by titration with 0.01 N $KMnO_4$ with a calculation coefficient of 0.58 (75 ml of distilled water, 1-2 ml of the solution to be titrated, 2.5 ml of indigo carmine solution); the results of the titration were used to calculate the degree of tannin oxidation in per cent.

Results of Estimations. It became obvious during the investigation that the leaves had to be incubated for several hours after irradiation at a specific temperature in order to achieve oxidation of tannins. Even after the leaves received a dose of one million r, there was no detectable oxidation of the tannins immediately after irradiation. The relation between the degree of tannin oxidation and the duration of incubation postirradiation at 30-33°, for leaves of various age, is shown in Fig. 2a. It will be seen from the figure that the curve representing the oxidation of tannins in the old leaves is the flatter of the two. This is due to the large differences between tea leaves of various ages in their tannin content and in the activity of the polyphenol oxidase in the leaves. The difference was most pronounced at 2.5 hours postirradiation and gradually disappeared subsequently.

The degree of tannin oxidation was also found to be dependent on the temperature at which the leaves were incubated after the irradiation. A comparison of irradiated and nonirradiated leaves, incubated for 5 hours under the same conditions, showed that the postirradiation incubation had a characteristic temperature optimum (Fig. 2b) whose position coincided with the temperature optimum of polyphenol oxidase activity: 30-33° [3]. This indicated that in the irradiated leaves the oxidation of tannins apparently proceeded enzymatically, and was not a direct effect of irradiation.

In order to confirm the enzymatic nature of the oxidative process in the irradiated tea leaves, some experiments were carried out in a nitrogen atmosphere. Since oxygen is obligatory for the activity of polyphenol oxidase, it could be expected that under anaerobic conditions the extent of tannin oxidation would be less than in air. The actual results confirmed this (Table 1).

The intensity of tannin oxidation in whole irradiated leaves was then compared with the same activity in leaf homogenates.

The experimental procedure was as follows: four leaf-discs were irradiated, and two of the four were then incubated for 5 hours at 30-33°, while the remaining two were ground in the apparatus shown in Fig. 1 and then incubated under the same conditions as the discs. Grinding of the leaves starts off the fermentation process, during which the tannin content falls. The tannin titer in the whole irradiated leaf fell also, but in the ground, irradiated leaf the degree of tannin oxidation was lower than in the whole, irradiated leaf:

	Nonirradiated, %	Irradiated, 239,000 r, %	Irradiated, 900,000 r, %
Whole leaf	100	47	52
Ground leaf	35-37	55	74

The results may be explained as follows: irradiation per se disrupts the internal organization of the leaf tissue, and at this stage the mechanical damage (grinding) can no longer produce an additional effect but, to the contrary, some inactivation of the enzyme was observed after the grinding of the leaf. In the additional experiment, involving the irradiation of ground leaf with 1 million r with subsequent incubation for 2.5 hours at 37°, the level of tannin also fell compared with the control (ground, nonirradiated leaf). It thus, follows that irradiation no longer had any effect on ground leaves.

It should be noted that the increase in irradiation dose did not bring about a more complete oxidation of the tannins. It will be seen from Fig. 3 that after x-irradiation with a dose of up to 1 million r, or with fast electron

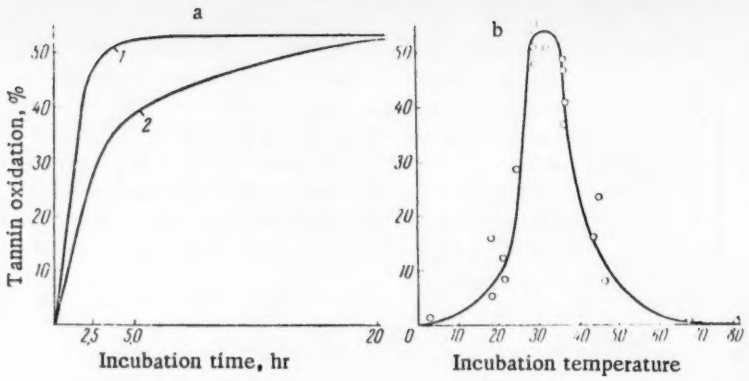

Fig. 2. The relation between the response to radiation and the duration of incubation and age of leaves (a) or the incubation temperature (b). a) Dose 0.5 million r; incubation temperature 30-33°; 1) young leaves ; 2) old leaves b) dose : 1 million r; incubation time, 5 hours; old leaves.

TABLE 1

Tannin Content of Irradiated Tea Leaves during Incubation in a Nitrogen Atmosphere (mg/ g) (incubation time — 2.5 hours at 30-33°)

Dose , thous. r	Nonirradiated leaves	Irradiated leaves		Tannin oxidation,%	
		in air	in nitrogen	in air	in nitrogen
500	53,8±1,2	28,2±2,1	40,7±2,2	48	24
500	59,6±0,3	34,6±0,7	49,0	42	18
900	58,7±5,7	28,1±0,2	36,6±1,0	52	38

irradiation (electron-accelerator) with doses up to 18 million rep, the limiting value of tannin oxidation did not exceed 50-60%. This result could also be explained by the limiting conditions of the enzymatic reaction, since the direct irradiative oxidation could be expected to proceed to completion.

Experiments with Tobacco Leaves

Experimental Methods. Leaves of tobacco of the "Trapezond 93" type, kiln-dried, of moisture content of 13% were used throughout. Eight discs, each weighing about 100 mg. were taken from one leaf; one half of this number were irradiated, the remaining serving as controls. Irradiations were carried out either with the BF tube or the electron-accelerator. The changes were assessed by their effect on the rate of loss of weight, or "dehydration" which usually takes place during the fermentation of tobacco leaves. This value represents the sum total of the biochemical changes of the contents and the physical properties of the raw material in the process of fermentation. In the present work these measurements were used in view of the established (published data) relationship between the extent of dehydration and the intensity of oxidative processes. Thus, according to Svirin [4], there is a parallel relationship between the oxygen consumption during tobacco processing and the dehydration over a wide range of temperatures (from 20 to 80°). Irradiated and normal discs were incubated together for 3-10 days at a given temperature and were then dried for 2.5 hours at 105°. The difference in weight loss between the normal and irradiated disc was used as an indication of the changes in the oxidative processes in the tobacco leaf as a result of irradiation.

Results. Preliminary experiments, involving the incubation of nonirradiated tobacco leaves at high temperatures, that is, under the conditions of ordinary thermal fermentation, showed that the difference in weight loss, compared to controls kept at 3°, was 0.7% after 7 days at 50° (13.0-12.7), and 5.3% (17.2-11.9) after 2 days at 70°; these data were the means of 2-3 experiments.

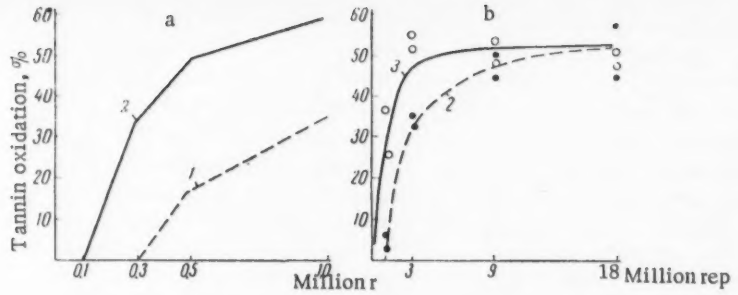

Fig. 3. The relation between the radiation effect and the irradiation dose with x-rays (a) of electrons (b). Postirradiation incubation at 30°. Duration: 1) 1 hour, 2) 2.5 hours, 3) 20 hours.

TABLE 2

The Effect of an Electron Beam on Tobacco Leaves

Exptl. conditions	Incubation days	Wet weight, mg	Dry weight, mg	"Moisture", %
Control	0	197,60	175,06	12,8
	3	239,86	211,93	13,2
	7	147,63	131,19	12,6
	10	174,77	155,75	12,8
Experimental	0	169,96	151,41	12,2
	3	149,92	132,91	12,8
	7	151,23	133,06	13,6
	10	156,14	134,21	16,4

Irradiated leaf-discs, and the nonirradiated controls, were incubated at 35°. When 1 million r was delivered to the irradiated discs, no difference in weight loss between the treated and control samples was observed even after a 10-day incubation period. When the dose was increased to 10 million r, a slight difference in weight loss about 1.6 % between the treated and control samples, was observed after the same incubation period. In view of the fact that the BF tube used could not deliver higher doses, subsequent experiments were carried out with the electron accelerator (dose rate 1.5 million rep/min); in this case the subsequent incubation was carried out at 50°. When the samples were irradiated for 15 min (dose, about 22 million rep), and subsequently incubated for 3 days, again no difference was found in the weight loss between the control and treated samples; a difference of about 1% observed when the incubation was allowed to proceed for 7 days; while after 10 days the difference reached 3.6%. Following 30 min irradiation (dose, about 45 million rep) and an incubation period of 10 days the difference in weight loss was 4.7 %, but under these conditions there was also some loss of water during irradiation (decrease of "moisture" to 10%), and this change somewhat slowed down the subsequent changes. Thus, high doses of irradiation (about 50 million rep) are required in order to effect a weight loss in tobacco and, thus, a change in the oxidative processes, of the same order (5-7%) as the values accepted in technology [4].

It follows from Table 2that immediately after irradiation with the electron beam for 15 min (dose, about 22 million rep), and also within the first three days postirradiation, the intensity of the oxidative processes in the irradiated samples differed little from those in normal controls, and only after 7-10 days were any changes observed. This circumstance could only by due to the fermentative nature of the oxidative processes, since the direct radiation-induced substrate oxidations, which has a local character, could be expected to be detectable in the very early stages, or even directly after irradiation.

DISCUSSION

The effect of radiation on living organisms may be generally considered as an example of a disturbance of a steady state of an open system whose distinctive properties have been discussed elsewhere [5]. The steady state

concentrations of substrates would obviously be affected not only as a result of substrate and enzyme interaction, but also through any change in the interralationship of these substrates, a change which may possibly arise as a result of altered diffusion or permeability, resulting from damage to intracellular structures. Thus Budnitskaya, Borisova and Pasynskii [6], who studied the effect of ionizing radiation (in doses of 10,000 r) on the substrates concerned with lipid metabolism and on the enzyme lipoxidase in a number of plants (soya, wheat, peas, etc.), showed that enzyme inactivation and nonenzymatic substrate oxidation were only of secondary importance in these processes. Moreover, in the immediate postirradiation period, some activation of the lipoxidase in the shoots of the investigated plants was detected, and at the same time the tissues became more permeable to electrolytes, as determined by conductivity measurements [17]. The presence of an initial activation period after irradiation is also in accordancewith the theory of open systems [5].

The present work dealt with the effect of ionizing radiation on the oxidative processes in tea and tobacco leaves. As mentioned above, it is these oxidative processes involving the polyphenols of tea and the glycosides of tobacco that determine the ultimate quality of the product. In tea processing these changes take place during the fermentation of tea through the action of enzymes, including polyphenol oxidase, whose effect is enhanced due to the preliminary bruising and maceration of the leaves which allows direct mixing of the enzymes and substrates and the possibility of direct reaction in the access of air. It was reasonable to assume that as a result of ionizing radiation, nonenzymatic oxidation of the tea-leaf tannins by the products of radiolysis was feasible. However, experimental results showed that the oxidation of tannins in the irradiated tea leaf was due to enzymatic processes, as evident from the following: 1) the oxidation of tannins did not take immediately after irradiation, as would have been expected in the case of oxidation by radiolysis products, but developed slowly over a number of hours after irradiation; 2) the optimal temperature for oxidation after irradiation corresponded to the optimal temperature of polyphenol oxidase activity; 3) the presence of gaseous oxygen, which is absolutely necessary for polyphenol oxidase activity, accelerated the postirradiation oxidation, while oxidation by radiolysis products would proceed at the expense of endogenous tissue oxygen; 4) the postirradiation oxidative process proceeded more rapidly in young leaves than in old ones. The oxidation of tannins in the irradiated leaf thus proceeds, apparently, enzymatically. It is however of interest to note that some oxidized forms of tannins do appear in the intact irradiated leaf, while in the normal leaf such accumulation does not occur unless the leaf has been bruised or macerated.

This thus allows the general conclusion that the effect of ionizing radiations on the whole leaf consists in some interference with the internal structure of the organized tissue, resulting in a more direct contact between various enzymes and their substrates, an effect similar to that observed as a result of bruising. The results clearly indicate that enzyme inactivation or direct substrate oxidation by the ionizing radiation can only be of secondary importance: the main factor appears to be the change of conditions under which the tannins may react, as a result of radiation damage to the tissue structures. These conclusions are of general interest in the elucidation of the mechanism of radiation effect on plant tissues. Similar conclusions may be arrived at from the experiments involving the irradiation of tobacco leaves with the electron beam, although in this latter case the developments were considerably slower due to the comparatively low "moisture" content of the fermenting tobacco leaves (ca. 13%) compared with that of tea leaves (70-80% in young leaves, 60-65% in old ones).

From the practical point of view, the utilization of ionizing radiations for the acceleration of tannin oxidation could not be justified at present because of the very high doses (5-50 million r) and high radiation intensities involved. However, the subject may merit reviewing when high energy radiation sources become commonly available.

SUMMARY

It was shown that oxidative processes in irradiated leaves were due mainly to enzymatic activity and not to a direct effect of radiation. Of considerable importance in the process is the damage to the internal organization of the leaf tissues, which affects the conditions of interaction between enzymes and substrates.

When tea leaves were irradiated for 10 min with powerful electron beams produced under a tension of 1 million volts and with a current of 1 ma, the degree of tannin oxidation typical for a usual fermentation (50-60%) was achieved without maceration or bruising.

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THE DISTRIBUTION OF L-ALANINE DEHYDROGENASE AND L-GLUTAMIC ACID DEHYDROGENASE IN MICROORGANISMS OF THE GENUS BACILLUS*

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The formation of alanine, glutamic acid, and phenylalanine from the corresponding α-ketoacids and ammonia by a cell suspension of Bac. subtilis (BS) has been described by Konikova et al. [1]. The results of experiments with acetone treated preparations of Bac. brevis [2] have shown the direct synthesis of alanine from NH_4+ and PA. In Brucella abortus AL is synthesized both by transamination and by direct aminaton of PA, according to the data of Altenbern and Housewright [3], and Marr et al. [4]. A more detailed study of the synthesis of AL in suspensions of washed BS cells was carried out by Fairhurst, King, and Sewell [5] who observed the energetic formation of AL from NH_4+ and PA when GL formation from NH_4+ and KG was very slight.

Further, Wiame and Piérard [6] showed the presence of AD-ase connected with DPN and acting reversibly in a cell-free extract of a mutant strain of BS which lacked GD-ase and required for growth the addition of GL or its immediate precursors: proline, ornithine, arginine, or aspartic acid (Am^- strain); the enzyme was purified and studied by Piérard [7]. Recently Jordan [8] observed that Rhizobium melliloti also synthesizes AL from PA and NH_4+ and has AD-ase. There are no satisfactory data on the ability of bacteria to synthesize monocarboxylic amino acids other than AL by direct amination. In order to establishe whether AD-ase plays a general part in some microorganisms in the assimilation of ammonia nitrogen like the role of GD-ase in most organisms, we had to discover the species and strains of bacteria which have AD-ase, and to study the amino acid metabolism in these organisms.

In the present investigations we showed the remarkable fact that the presence of AD-ase in connection with the absence of GD-ase is a general sign of all the normal ("wild") strains of BS studied by the authors, and of other species belonging to the genus Bacillus except Bac. anthracoides, in which AD-ase and GD-ase are present at the same time.

MATERIALS AND METHODS OF STUDY

Bacterial Strains. Cultures of Bac. subtilis N-1235, Bac. mycoides, N-537, Bac. mesentericus. N-1226, Bac. megatherium LI-654, Bac. brevis, Bac. anthracoides N-1312 were obtained from the museum of live cultures of the control Institute for Sera and Vaccines of the Ministry of Health of the USSR. Bac. subtilis, strains B-1, N-83, and N-9789 were supplied by the A. N. Bakh Institute of Biochemistry, Academy of Sciences, USSR and the Institute of Microbiology, Academy of Sciences, USSR. BS Z-1, a "wild" strain, was freshly isolated from air by Yu. I. Rubinshtein (Institute of Nutrition, Academy of Medical Sciences, USSR). Strains BS IR C-1 and IR C-7 (Am+ mutants) were kindly sent by J. Wiame (Belgium)***. They were a variant of Am^- mutants which again had

* Abbreviations in text: AL) alanine; GL) glutamic acid; AD-ase) L-alanine dehydrogenase; GD-ase) L-glutamic acid dehydrogenase; PA) pyruvic acid; KG) α-ketoglutaric acid; DPN) diphosphopyridine nucleotide; TPN) triphosphopyridine nucleotides; BS) Bac. subtilis.

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*** We take the opportunity to express thanks to Professor J. Wiame and the Institutes mentioned for supplying the cultures and information on the characteristics of the strains.

TABLE 1

Formation of Alanine from Pyruvate and Ammonium Ions in Washed Cells of Bac. subtilis N-1235. Composition of sample: 1 ml of cell suspension (28 mg dry weight of bacteria); 100 μmoles PA or KG; 50μ moles NH_4Cl ; 0.05 M phosphate buffer (pH 7.5). Total volume 2.5 ml. Incubation 3 hours at 37°

Sample	AL	GL
	μmoles in sample	
Before incubation	2,5	1,9
Without substrate	2,5	4,9
With NH_4Cl	1,6	1,9
" PA	6,8	1,3
" PA and NH_4Cl	14,0	1,6
" KG	Следы	3,7
" KG and NH_4Cl	0,9	3,4

the power to assimilate NH_4+. This strain contained GD-ase and lacked AD-ase activity.

Nutritive medium and conditions of growth. Depending on the problem under investigation the cultures were grown on ordinary broth or on a synthetic medium containing 1% glucose; 1% L-Gl (Na salt); 0.1 % K_2HPO_4; 0.05% $MgSO_4 \cdot 7H_2O$ 0.01% NaCl; 0.001% $FeSO_4$; and 0.01% $CaCl_2$; the pH was adjusted to 6.9-7.1. The nutritive medium was poured in portions of 50 ml into 500 ml Erlenmeyer flasks, seeded with the culture to be studied, washed from slanted agar, and then the flask was incubated at 37° on a shaker operating at 200 rpm. The bacterial cells for preparing the suspension were collected by centrifuging during the period of exponential growth.

After washing three times with distilled water, the cells were suspended in a definite volume of 0.05 M phosphate buffer so calculated that 1 ml contained about 20 mg of dry weight of bacteria. The density of the sample was determined by measuring the cloudiness in a photoelectric colorimeter and calculating from a graph calibrated with a definite dry sample of bacteria.

Cell-free preparation. The bacterial cells were lysed with lysozyme by the process described by Nomura et al. [9]. The cells collected in the exponential phase of growth, were suspended in 0.01 M phosphate buffer (pH 6.8); for lysis we added about 1 mg of crystalline lysozyme for each 100 mg of dry weight of bacteria. The mixture was incubated for 1 hour at 35°, stirred slightly, and then centrifuged 40 min at 25,000 rpm. The resulting clear supernatant liquid was dialyzed and used for studying the enzyme activity. In order to obtain an ultrasonic extract, we suspended the washed bacterial cells in 0.005 M phosphate buffer (pH 7.5) and carried out disintegration in an ultrasonic generator at twelve kilocycles for 40 min at 4° . The resulting suspension was centrifuged at 25,000 rpm and the residues was discarded.

Measurement of enzyme activity and chemical analysis. The activity of AD-ase and GD-ase was measured by determining the increase in optical density at 340 mμ due to reduction of DPN or TPN by the corresponding amino acids; the measurements were made in the SF-4 spectrophotometer. Protein was determined by the quantitative biuret method [10]. Free amino acids (AL, GL, glutamine) were determined quantitatively by paper chromatography [11] after single phase separation in a mixture of butanol-formic acid-water (75:15:10). Total amino acid nitrogen was determined according to Cocking and Yemm [12], and PA was determined as the 2,4-dinitrophenylhydrazone [13].

RESULTS

Synthesis of AL in washed cells of Bac. subtilis. In most of the experiments we used as the source of cells the culture of the normal strain BS N-1235 grown on agar broth for 8 hours. The cells were collected during the period of exponential growth , washed, and incubated in the centrifuge tube with substrates PA with NH_4Cl or KG and NH_4Cl.

We found that in the presence of ammonium salts a sufficiently large amount of AL was formed from PA, while GL was not formed from KG (Table 1). PA in the absence of NH_4Cl produces increase in AL with simultaneous disappearance of previously formed GL.

The results of these experiments and analogous ones carried out with other BS strains agree with the data of the investigation of Fairhurst et al. [5].

In order to find out whether AL is formed from PA by direct reductive amination, or is the result of transfer of an NH_2 group from another primary acceptor of NH_3, we ran the following experiments. The bacterial sample was incubated with a mixture of substrates: PA + NH_4Cl, PA + GL and PA + glutamine. After we had precipitated the protein by boiling, we determined on a paper chromatogram the content of AL, GL, and glutamine in the fil-

TABLE 2

Amination of PA from a Cell Suspension of Bac. subtilis N-1235 in the Presence of NH_4^+, GL, and Glutamine. Composition of incubated mixture and conditions of experiments, see Table 1; dry weight of bacteria in each sample 25 mg. Glutamine and GL added in amounts of 18 μ moles per sample

Sample	Alanine	Glutamine	Glutamic acid	Nonprotein amino N
	Found in μ moles for the test			
Without incubation				
PA+ NH_4Cl	3,4	0	2,6	8,5
PA+ L-glutamine	2,6	18,4	2,6	26,0
PA+ L-Gl	2,8	0	17,9	21,0
After 3 hours incubation				
Without substrate	6,5	0	6,2	12,5
NH_4Cl alone	6,5	0	5,3	8,0
L-Glutamine alone	6,8	8,0	13,6	32,0
L-Gl alone	6,4	0	17,9	29,0
PA alone	9,8	0	2,6	15,0
PA + NH_4Cl	29,3	0	3,1	33,5
PA + L-glutamine	20,7	11,1	12,4	38,5
PA + L-Gl	9,6	0	17,9	30,5

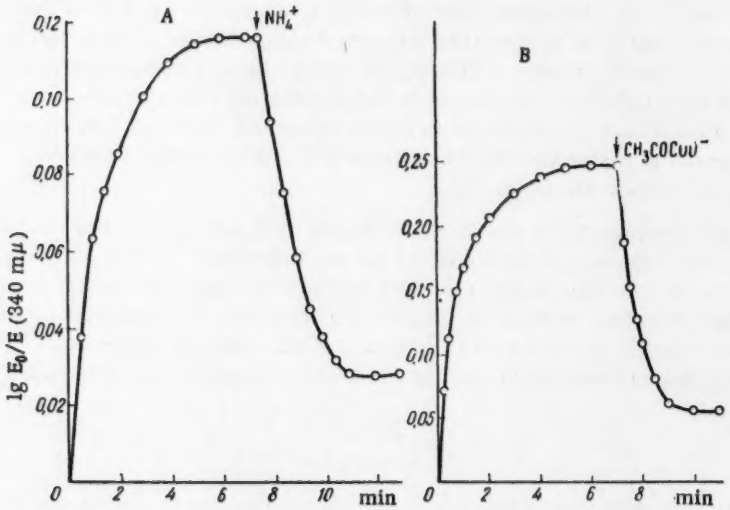

Fig. 1. Course of the alanine dehydrogenase reaction with time and its reversibility. Experimental conditions: dialyzed lysozyme extract of BS N-1235 which contain in experiment A 2.7 mg of proteins, in experiment B 4.7 mg, incubated at 24° with 10 μ moles L-AL, 0.6 μ moles DPN, and 1.5 ml of pyrophosphate buffer (0.1 M; pH 8.3) in a total volume of 3 ml. At the moment indicated by the arrow we added 40 μ moles NH_4^+ (A) or 20 μ moles PA (B).

trate. The results of the experiments were the same: only a very small amount of AL was formed from GL and PA no more AL than was formed from PA alone due to transamination with endogenous amino acids. When glutamine was used instead of GL we found a considerable increase in AL (11 μ moles) which was equivalent to a decrease in glutamine (of 9 μ moles) and also an increase in GL (10 μ moles) and total amino nitrogen (12 μ moles). This shows

TABLE 3

Balance of the Reaction Catalyzed by L-Alanine Dehydrogenase. Composition of reaction mixture: enzyme solution (2.2 mg protein), 16 μmoles L-AL, 1.6 μmoles DPN, 1 ml 0.01 M pyrophosphate buffer (pH 8.3), total volume 3.0 ml, temperature 24°

Reaction component	Before incubation, μmoles	After 23 min incubation, μ moles	Δ, μmoles
AL	16,0	15,86*	—0,14
DPN+	1,6	1,45**	—0,15
PA	0	0,14	+0,14
DPNH	0	0,15	+0,15

* Calculated on amount of PA formed.
** Calculated on amount of DPNH formed.

that for the amination of PA there was used the ammonia liberated in the enzymatic hydrolysis of glutamine in the BS cells, while no sort of transamination with glutamic acid transaminase occurred.

Activity of AD-ase. The results of the experiments which we carried out and the literature data [5-7] gave a basis for assuming that the transformation of PA into AL occurred chiefly under the influence of a specific AD-ase. The activity of this enzyme was studied in six strains of "wild" type BS. The bacteria were grown in a synthetic medium for 20 hours, separated during the period of exponential growth, and treated with lysozyme. The cell-free extract was dialyzed and studied for dehydrogenase activity.

In the extracts from the cells of all strains it was easy to detect the presence of AD-ase by its action on DPN (TPN is not reduced), and to show the reversibility of the reaction. The results of the experiments with extracts from BS N-1235 are shown in Fig. 1; with extracts from the other strains analogous results were obtained.

Balance of reaction catalyzed by AD-ase. The cell-free extract from BS N-1235 was precipitated by 75% saturated ammonium sulfate and the precipitate was dissolved in 0.05 M phosphate buffer at pH 7.5. After centrifuging and dialysis we obtained the enzyme fraction used for the experiment.

The samples which contained L-AL and DPN were incubated at room temperature (24°) in a spectrophotometric cuvette; we determined the change in optical density at 340 mμ before reaching full equilibrium, and after this, in the equilibrium liquid we determined the PA content. As Table 3 shows, 0.14 μmoles of PA and 0.15 μmoles of reduced DPN (DPNH) were formed, corresponding with a theoretical balance of reversible dehydrogenation of alanine according to the equation $CH_3CH(NH_3^+)COO^- + DPN^+ + HO \rightleftharpoons CH_3COO^- + DPNH + H^+ + NH_4^+$.

The equilibrium position of the reaction (Table 3) reflects the great predominence of the synthesis of AL over its deamination [7].

GD-ase acitivity. In analogous experiments with L-GL as the substrate and with DPN or TPN as the hydrogen acceptor we showed the complete absence of GD-ase activity in the lysozyme lyzate and in extracts of the ultrasonic-treated cells of all the studied strains of BS including strain Z-1 freshly isolated from the external medium (Table 5). The negative results were reproduced in repeated studies on the activity of GD-ase in BS cultures under different conditions.

The studies on the activity of AD-ase and GD-ase were also made on extracts of cells of the two Am^+ mutants (strains IRC-7 and IRC-1) obtained from the laboratory of Prof. Wiame. In the cell free extracts of these bacilli it was easy to detect the activity of GD-ase with TPN as coenzyme (Fig. 2), corresponding to the results of the Wiame school.

The same extracts were shown to be lacking in AD-ase activity, which was somewhat unexpected, since in the work published by Wiame and co-workers there was no evidence of absence of AD-ase in the BS mutants transformed from Am^- into Am^+ forms.

Transamination reactions in BS. The results given in Tables 1 and 2 showed that the intensity of the transamination between L-GL and pyruvate in undamaged BS cells (normal strain) was very small.

When cell free extracts were used as the source of the transaminase, the reaction rate of transamination between PA and α-amino acids (both mono- and dicarboxylic) was lower than the rate of the analogous reaction between KG and α-amino acids (Table 4). The amount of AL formed in transamination between PA and D-GL is three to four times higher than in the reaction with L-GL; correspondingly, from DL-AL and KG there is produced

TABLE 4

Comparison of Transamination Reaction Rates between α-Amino and α-Ketoacids in Preparations from Bac. subtilis N-1235 (in μ moles in the sample). Reaction mixture: 50 αmoles PA or KG; 50 αmoles acid; 20 αg pyridoxal phosphate (not added in the experiment with acetone treated powder); 1.0 ml 0.05 M phosphate buffer (pH 7.9). Enzyme preparations: in experiment I) dialyzed lysozyme lyzate (2.8 mg of protein); in experiment II) dialyzed extract of ultrasonic treated cells (3.0 mg of protein); in experiment III) 20 mg of bacterial cells treated with acetone. Total volume of sample 2.5 ml. Incubation 2 hours at 37°

Sample	Experiment I		Experiment II		Experiment III	
	AL	GL	AL	GL	AL	GL
Without incubation (control)	0	0	0	0	Trace	1,3
Incubation:						
Without substrate	0	0	0	0	Trace	0
PA alone	0	0	0	0	0,6	1,3
PA + L-GL	2,3		1,1		2,7	—
PA + D-GL	9,7		4,9		—	
PA + DL-leucine	1,3		1,1		—	
PA + DL-valine	2,0		1,4		—	
PA + DL-AS	1,3		1,4		—	
KG alone	0	0	0	0	Trace	1,5
KG + DL-AL		9,3		9,7		12,0
KG + DL-AS		1,0		3,0		10,0
KG + DL-leucine		4,0		3,5		—
KG + DL-valine		4,7		3,8		—

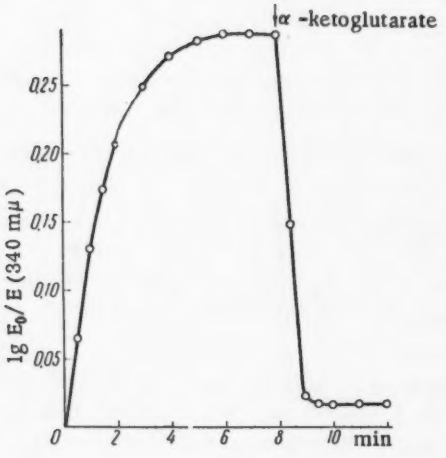

Fig. 2. Activity in relation to TPN of glutamic acid dehydrogenase in cell-free extracts of BS IRC-1 (Am^+ mutant) The dialyzed lysozyme extract contained 5.9 mg of protein. We introduced into the sample 20 μ moles of L-Gl and 0.6 μ mole of TPN; the arrow indicates the moment of adding 20 μ moles of KG. (Analogous results were obtained with extracts from the cells of mutant IRC-7.)

a considerable amount of GL. This finding confirms the earlier observation of Thorne et al. [14,15] who showed that in BS the activity of D-GL-D-AL transaminase as appreciably higher than the activity of the individual L-transaminases. Aside from the D-AL transaminase in BS and other forms of the genus Bacillus there is a specific alanine racemase [14]; therefore L-AL was not used as a starting substrate for studying the transamination reaction in bacilli which takes place with participation of this isomer. The D-isomers of other amino acids (except AL) do not transaminate with KG in BS preparations [14,15]. Therefore, the values for the transformation obtained in samples with KG and DL-leucine or DL-valine on the one hand and in samples with DL-GL and PA on the other permit us to conclude that in BS, KG undergoes transamination with L-valine and L-leucine more actively than with L-AL (Table 4). The presence of very active GL-AL-transaminase can be observed only in experiments with acetone treated preparations of bacterial cells, but not with the extracts. These results on the relative intensities of various transamination reactions in BS N-1235 agree with the results of analogous experiments carried out by a number of authors on other strains of this microbe [5-7, 14-16].

Distribution of AD-ase and GD-ase in several forms of the genus Bacillus. The results of the above experiments which show the absence of GD-ase and the simultaneous presence of AD-ase in a series of normal strains of BS permit us

TABLE 5

Distribution of L-Alanine Dehydrogenase and L-Glutamic Acid Dehydrogenase in Various Strains of Bac. subtilis and Other Types of Bacillus. As unit activity of AD-ase or GD-ase we took that amount of enzyme which in the spectrophotometric test under the conditions given in Fig. 1 caused a change in optical density (ΔE_{340} mμ) of 0.001 per min (in the first 30 second interval). The specific activity corresponds to the number of activity units per 1 mg of protein in the tested unpurified lysozyme extract of the bacterial cell

Type and strain of Bacillus	Specific activity	
	AD-ase	GD-ase
Bac. subtilis N-1235	42,6	0
B-1	18,0	0
Z-1	23,1	0
IRC-1 (Am^+-mutant)	0	30,5
IRC-7 (Am^+-mutant)	0	17,3
[*Bac. subtilis*, IRC-2, IRC-6 (Am^--mutant[13,16])	[+]	[0]
Bac. mycoides N-537	18,0	0
Bac. mesentericus vulgatus N-1226	38,8	0
Bac. megaitherium Ll-654	44,5	0
Bac. brevs	20,6	0
Bac. anthracoides N-1312	57,1	108,6
[*Bac. cereus*][17]	[+]	?

to assume that in these microorganisms the first act in the assimilation of ammonia nitrogen is the amination of PA. These results led us to investigate the distribution of AD-ase and GD-ase in other forms of spore-bearing aerobes. We studied, in addition to BS, five examples of the genus Bacillus, namely: Bac. mycoides, Bac. mesentericus vulgatus, Bac. megatherium, Bac. brevis, and Bac. anthracoides. Cell-free lysozyme lyzates were prepared from cells of these microbes, taken during the exponential phase of growth, as described above. Spectrophotometric study showed that the cell-free extracts of all these organisms had AD-ase activity of the same order as the BS extracts.

According to a personal communication from Prof. Wiame, in his laboratoly independently of our observations, he found the presence of AD-ase in cells of all the studied forms of bacilli and some otherGram-positive bacteria. Our results and those of the Wiame group agree with the finding of Fairhurst et al. [5] according to which a cell suspension of most Gram-positive bacteria, including several form of the genus Bacillus, from AL more or less actively from PA and NH_4^+.

It is an especially interesting fact that all these studied types of Bacillus, like BS show a lack of GD-ase, except for Bac. anthracoides, in the extracts of which we found a high activity of both AD-ase and GD-ase. The only other exception known at the present time is the presence of GD-ase in the Am^+-mutants of BS (strain IRC-1 and IRC-7) which come from the Am^- strain and, as noted above, do not have AD-ase.

The results of our investigation on the distribution of AD-ase and GD-ase in different strains and types of Bacillus are summarized in Table 5 where also give (in parenthesis) some of the related data of Wiame and his co-workers [6,7].

DISCUSSION OF RESULTS

It has been established that AL is synthesized by a suspension of BS cells [2,5] and appears in the culture liquid when this microbe is grown on a synthetic medium. It has been found that L-Al plays the part of an activator in the growth of the spores of Bac. terminalis [18]. The reaction catalyzed by the specific alanine racemase and D-AL-KG transaminase are steps in the process of synthesis of D-GL by cells of Bac. subtilis and related types[14], which form extracellular D-glutamyl polypeptides. The combination of the results given in our paper and those published earlier by Fairhurst et al. [5] and by Wiame and co-workers [6,7,16] together show that the synthesis of

Al (first the L-isomer) in normal strains of Bac. subtilis and a number of other types of Bacillus occurs chiefly by direct reductive amination of PA.

The primary role of the synthesis and transformation of AL in the nitrogen metabolism of microorganism which belong to the genus Bacillus is still further emphasized by the presence in these organisms of AD-ase, as shown in the present work and established independently of us by Wiame, and especially by our observation of the absence of GD-ase in all normal strains of BS and in a number of forms of Bacillus. The only exception is Bac. anthracoides which contains both AD-ase and GD-ase.

This fact allows us to assumethat the amination of PA catalyzed by AD-ase may be the chief initial reaction of ammonia assimilation in many forms of the genus Bacillus and perhaps in some other gram positive bacteria [3-5]. This point of view is confirmed by a number of experimental results given by the authors in another communication [17].

AD-ase was discovered by Wiame and Piérard [6,7] in the cells of a mutant BS which had lost the ability to assimilate ammonium ions and required for their growth the addition of GL or its immediate precursors (Am^- strain). Wiame and his school connected the block in assimilation of ammonia with the absence of GD-ase in these mutants; they did not succeed in explaining the fact that the Am^- strain of BS also lacked the ability to use for their growth the nitrogen of L-AL or L-leucine, although they could form GL from the amino acids mentioned by transamination.

The data described in the present work showed very clearly that the absence of GD-ase is not a special defect of the enzyme apparatus of Wiame's Am^- mutant, but a normal biochemical property of many existing forms of the genus Bacillus. This certainly does not mean that the ancestors from which these strains came had this characteristic. Study of the "wild" types of the bacillus showed that they grew well on synthetic media which contained suitable sources of carbon and ammonium ions as the sole source of nitrogen nutrients [17]. Hence, the inability of the Am^- strain of Wiame to assimilate ammonia cannot be explained by the absence of GD-ase; it must depend on other metabolic defects, the nature of which remains to be explained.

It is also evident that the transformation of the Am^- mutant into the Am^+ for which assimilates ammonia (for example, the strains IRC-1 and IRC-7) and which sometimes occurs in cultures of the Am^- strains under special conditions is not the result of a "reverse" mutation with reversion to the "wild" type. The Am^+ strain of Wiame is certainly an anomalous product of a secondary mutation (and selection) which results in the renewed appearance of the ability to elaborate GD-ase (an enzyme which is not normally in BS) with simultaneous loss of activity of AD-ase, an enzyme which is normally a taxonomic sign of the genus Bacillus and which is retained in the maternal Am^- BS mutant.

At the present time GD-ase is accepted as a necessary participant in the nitrogen metabolism of all living cells. The complete absence of this enzyme shown in the present study in a large group of widely distributed amino autotropic microorganisms presents an unexpected and very interesting problem. It will certainly be necessary to study this in connection with the genetic plan of these microorganisms, and with their evolutionary position and the comparative biochemistry of their nitrogen metabolism.

SUMMARY

A cell suspension of normal ("wild") strain of Bac. subtilis easily forms alanine by amination of pyruvic acid, but does not aminate α-ketoglutaric acid. For the synthesis of alanine, aside from ammonium salts, the amino group of glutamine is used (by virtue of the presence of glutaminase in the cells of Bac. subtilis). The cells of Bac. subtilis do not carry out transamination between L-glutamic acid or pyruvic acid on any large scale.

The cells of all normal strains of Bac. subtilis and five other forms which we studied and which belong to the genus Bacillus, contain the L-alanine dehydrogenase, which acts with the participation of DPN, and which was found earlier by Wiame and Pierard in the Am^- mutant of Bac. subtilis, which lacks the L-glutamic acid dehydrogenase and does not assimilate ammonia nitrogen.

It is shown that the absence of L-glutamic acid dehydrogenase is a general property of all strains of Bac. subtilis and other forms of the genus Bacillus studied, except for Bac. anthracoides in which L-alanine and L-glutamic acid dehydrogenases are present together.

In the two "secondary" Am^+ mutants of Bac. subtilis (Wiame), which again have the ability to asimilate ammonia and have L-glutamic acid dehydrogeanse, L-alanine dehydrogenase cannot be found.

Amination of pyruvic acid under the action of L-alanine dehydrogenase is evidently the chief initial reaction in the process of assimilation of ammonia nitrogen in Bac. subtilis and in many other forms of the genus Bacillus. The inability of the Am^- mutant of Bac. subtilis to fix ammonium salts depends not on the absence of L-glutamate dehydrogenase, but on other biochemical defects, whose nature reamins unknown.

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A STUDY OF THE MACROMOLECULAR STRUCTURE OF THE INFECTIOUS RIBONUCLEIC ACID FROM TOBACOO MOSAIC VIRUS

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We showed previously [1] that when we raised the temperature of a solution of a native highly polymerized ribonucleic acid (RNA) from tobacco mosaic virus (TMV) which had infectious activity, its macromolecule underwent a structural change which was shown by a sharp increase in its special asymmetry. On the basis of the results of measuring the viscosity and sedimentation, we concluded that under these conditions, the polynucleotide chain passed from a coiled state to a straightened one. Since this change occurred at lower temperatures in the presence of urea and was shown more clearly than in an ordinary phosphate buffer, we suggested that the chief role in this effect was played by rupture of hydrogen bonds. This change was reversible and did not cause inactivation (loss of infectiousness) of the RNA [1]. It seems interesting to study this effect further using different physicochemical methods; the present study is devoted to this.

MATERIALS AND METHODS

For the preparation of RNA we used a TMV from infected tobacco leaves collected at the Abkazkian Tobacco Station in the summer of 1958. Isolation of TMV from the leaves and its purification were carried out by the method described before [2]. The preparation of the RNA from the TMV was carried out immediately before the experiments, by phenol deproteinization [2] with the exception that the time of deproteinization was increased to 20 min. The concentration of the virus RNA in solution was determined spectrophotometrically after hydrolysis [2]. Measurement of viscosity was carried out in an Ostwald viscosimeter with an outflow time of about 300 sec at 20° for water. Sedimentation was carried out in an ultracentrifuge of the Five system at 40,000 rpm. Measurements of optical rotation were made at 589 mμ (D line of sodium) in a polarimeter with a thermostatically controlled cuvette of length 22 cm (volume about 15 ml). Light scattering was measured at angles of 90, 45, and 135° with a Pulferich photometer. The solutions were freed from dust by centrifuging for 30 min at 15,000 rpm.

RESULTS

Properties of RNA at Room Temperature

At the present time a number of studies have been made of the physicochemical properties of infectious RNA from TMV in solutions at lower temperature, about 5° [3-5]. We, however, have made measurements at 20°. Our data on the sedimentation at 20° in a phosphate buffer with ionic strength 0.05-0.1 (pH 7.2) agree in general with those in the literature [4,5]. The sedimentation constant extrapolated to infinite dilution is 30-33 Svedburg units (Table 1). The characteristic viscosity for different preparations in phosphate buffer with ionic strength 0.1 is 120-180 cm^3/g (Table 2 and [2]).

In a 6 M urea solution with ionic strength 0.1 these values for infectious RNA change slightly, but all the

same there is a definite tendency toward increased viscosity and decreased sedimentation constant (calculated to standard conditions - water 20°, $[\eta] = 150\text{-}240$ cm^3/g; $S_{20w} = 27$ Svedberg units). This is evidently because of some disintegration in the RNA macromolecule as a result of partial rupture of hydrogen bonds by urea, as detected earlier by spectrophotometric measurements [6].

If the viscosity and sedimentation characterize the dimensions and form of the particles, the specific optical depends on the regularity of the macromolecule structure. The nucleotides themselves, which make up the polynucleotide chains, have only slight optical rotation, while for solutions of the infectious RNA at room temperature the value of the specific optical rotation is appreciable and at 589 mμ equals + 180°, at 546 mμ, +210°, and at 436 mμ, + 400° [4]. It was shown that enzymatic hydrolysis of virus RNA leads to a sharp fall in optical rotation of the solution, to a value on the order of + 20° [4]. All this shows that under these conditions in highly polymerized infectious RNA from TMV almost the whole optical activity depends, not on the asymmetrical atoms of its nucleotides, but on the spirals, the regular arrangement of the nucleotide residues in the macromolecule. Such a spiral arrangement of the monomeric units in the polymer chain (the so-called secondary structure) produces the strong optical rotation which is found in polypeptides and proteins.

According to our measurements, the specific optical rotation of infectious RNA $[\alpha]_D^{20}$ (in a phosphate buffer with ionic strength 0.1 at pH 7.2) is +170°. In recently published work of Doty and co-workers [8] the value of $[\alpha]_D^{20}$ in a phosphate buffer is also +170°. This establishes the value of the regular structure in RNA.

On the other hand, a whole series of observations show that the macromolecule of native RNA is not completely organized into a double-chained, rigid structure of the type of native DNA, but is an ordinary polynucleotide chain, rolled up in solution into an irregular ball which is reversibly altered under the action of different conditions without loss of infectiousness [5,6]. However, this does not exclude the possibility of the existence within the macromolecule of separate spiral portions as is indicated by the considerable optical activity.

When a solution of infectious RNA is treated with 6 M urea, keeping the same ionic strength (0.1), the optical rotation falls somewhat, $[\alpha]_D^{20}$ of infectious RNA in 6 M urea at $\mu = 0.1$ is +145°. The decrease in optical rotation of RNA in the presence of urea suggests the decisive role of hydrogen bonds in creating an ordered structure in the RNA macromolecule which is responsible for its optical activity. It is interesting to observe that 6 M urea does not affect the value of the infectious activity of the RNA and does not cause its inactivation [1,6]. Hence the easy breakdown of the structure which accompanies some changes in the tertiary structure (as shown by viscosity and sedimentation) and in the secondary structure (as shown by optical rotation) of the infectious RNA does not affect its biological activity.

Measurement of the molecular weight by the method of light scattering was carried out by us in 6 M urea solutions at ionic strength 0.1; they showed no change in the molecular weight of the infectious RNA as compared to the data in the literature relating to ordinary water and buffer solutions of RNA from TMV [5,9]. The molecular weight of the macromolecule of infectious RNA in a 6 M urea solution also is close to $2 \cdot 10^6$ (Table 3). Analogous values are obtained in calculating the molecular weight of infectious RNA in 6 M urea from our data on viscosity and sedimentation if we start from the idea that this polymer, at room temperature, is characterized by a macromolecule of the type of a rigid extended ellipsoidal rotation [4].

If we consider the RNA molecule as a rigid, cylindrical rod, then its length L, from light-scattering data, is 1600-2200 A (Table 3). At a molecular weight of $2 \cdot 10^6$ the diameter of the macromolecule, D, calculated by the formula for the volume of a cylinder $v = \frac{M}{d} = \frac{\pi}{4} LD^2$ (at $d = 1.67$ g/cm^3) is 27-31A. Hence, if we round off L=2000 A, and D = 30 A, then L/D = 70. An analogous value is obtained in calculating a/b from the data on characteristic viscosity in 6 M urea with ionic strength 0.1 (Table 1) according to the table of Simha [10] for rigid extended ellipsoidal rotation (rods). If $[\eta]$ is 150-240 cm^3/g, then $\nu = [\eta] \cdot d$ corresponding to 250 to 400, from which a/b = 60-80.

This value of L/D for an extended rod corresponds to the value of the constant β in the equation of Scheraga and Mandelkern which is $3.1 \cdot 10^6$ [11]. When we use $\beta = 3.1 \cdot 10^6$ for calculating the molecular weight from the data of viscosity and sedimentation in 6 M urea with $\mu = 0.1$, we also obtain a value on the order of $2 \cdot 10^6$.

Hence, in 6 M urea at $\mu = 0.1$ and room temperature the infectious RNA, just as in an ordinary buffer medium, consists of compact-folded polynucleotide chains and is an extended rod-shaped macromolecule with respect to the axes a/b = 60-80 and molecular weight $2 \cdot 10^6$, with a secondary and tertiary structure.

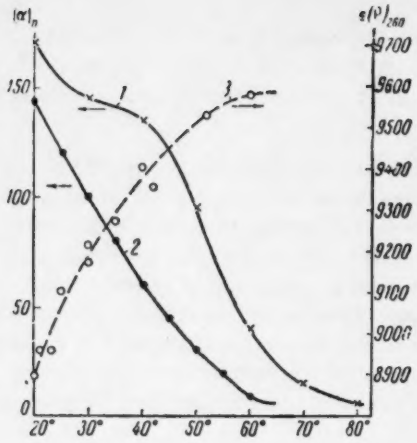

Fig. 1. Change in specific optical rotation and ultraviolet absorption of RNA solutions depending on temperature. 1) Optical rotation in a phosphate buffer, pH 7.2 with μ=0.1; 2) optical rotation in 6 M urea, buffered by phosphate to pH 7.2 and μ = 0.1; 3) ultraviolet absorption in 6 M urea at the same pH and ionic strength.

Change in Properties of RNA in Solution at Raised Temperature

As we have already mentioned before, heating the solution of infectious RNA causes a sharp change in its macromolecular properties which evidently include a change from the compact to the more straightened state of the polynucleotide chain. Apparently this change is connected with rupture of the hydrogen bonds [1].

The change in optical rotation of RNA depending on the temperature of the solution both in a phosphate buffer with pH 7.2 and μ = 0.1 and in 6 M urea at the same pH and ionic strength is shown in Fig. 1. In the case of 6 M urea with μ = 0.1 there is also a change in ultraviolet absorption at 260 mμ. From these results it is seen that with rise in temperature there is an intensification of ultraviolet absorption, that is, a rupture of hydrogen bonds. Detailed study of ultraviolet absorption depending on the temperature of the solution of infectious RNA can be found in the work of Boedtker [5]. Along with rupture of the hydrogen bonds in RNA there is a fall in optical activity; that is, destruction of the organized part (secondary structure) of the macromolecule. The presence of urea permits destruction of the secondary structure (Fig. 1). If the maximum fall in optical rotation in the phosphate buffer occurs at 80°, then in 6 M urea it occurs even at 60°. This once more confirms that the secondary structure which is responsible for the optical rotation of infectious RNA is maintained by hydrogen bonds. When the solution of infectious RNA is heated there occurs a gradual rupturing of the hydrogen bonds between the nucleotides within the macromolecule and a destruction of the oriented (spiral) particles.

In the work of Doty et al. [8] there are given analogous results on change in optical totation of infectious RNA in phosphate buffer depending on temperature. The action of temperature on optical rotation is considered by these authors as the results of "melting" of the spirals due to rupture of hydrogen bonds [8]. Similar melting of spiral secondary structures was studied earlier in proteins, also using measurement of optical activity [13].

Rupture of the hydrogen bonds and destruction of secondary structures of the macromolecule are not the only occurrences; there is change in the tertiary structure; that is, unfolding of the polynucleotide chain as a whole, since this latter should depend on other factors, the electrostatic repulsions of the phosphate groups. For a study of the change in form of the macromolecules we investigated the specific viscosity when solutions of different concentrations of RNA were heated.

It has been shown that in dilute RNA solutions a regular change in viscosity occurs just as in more concentrated solutions [1]; that is, the structural change at raised temperature is accompanied by considerable increase in specific viscosity. However, as Fig. 2 shows, the lower the RNA concentration, the lower will be the temperature at which this rise in specific viscosity begins, and the wider will be the temperature range in which it occurs, both with a phosphate buffer, pH 7.2, μ=0.1 (Fig. 2a) and with a 6 M urea solution at the same pH and ionic strength (Fig. 2b). The presence of urea lowers the temperature of the change and produces a more clearly shown rise in specific viscosity, as already mentioned previously [1].

We have assumed that the relation of the course of the curve to the RNA concentration is due to the polyelectrolyte effect, that is, the effect of the concentration of the opposite sodium ion. With a greater concentration of RNA in the solution there is also present a greater concentration of opposite ions (sodium or potassium cations) which screen the phosphate groups of RNA and thus impede the straightening of the polynucleotide chain at raised temperatures. At lower RNA concentrations the electrostatic repulsion is shown strongly which results in an early straightening of the chain, when not all the hydrogen bonds have yet been broken by the heat motion, and a lengthened interval of transformation, since further unfolding of the chain occurs gradually along with rupture of the hydrogen bonds.

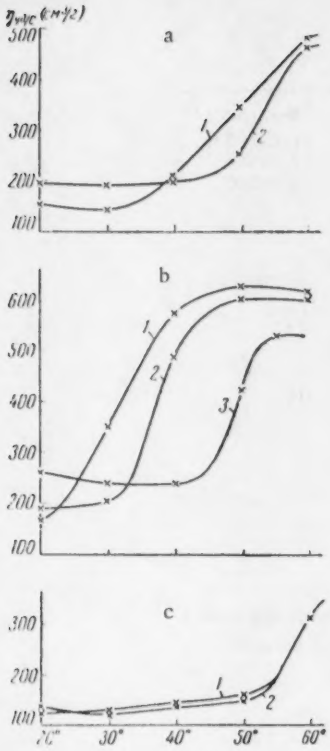

Fig. 2. Change in specific viscosity of RNA solutions depending on temperature. 1) RNA concentration 0.002 %; 2) RNA concentration 0.1%; 3) RNA concentration 0.25%; a) RNA solution in phosphate buffer pH 7.2, μ = 0.1; b) RNA solution in 6 M urea, pH 7.2 μ = 0.1; c) RNA solution in 6 M urea, pH 7.2, μ = 0.6 .

In order to show the correctness of this explanation, we used along with the 6 M urea solution at μ = 0.1 a 6 M urea solution with μ = 0.6 (phosphate buffer, pH 7.2 μ=0.1 with addition of 0.5 M NaCl). The data given in Fig. 2c show that with increase in concentration of electrolyte even at low RNA concentration, the increase in specific viscosity begins later and is concentrated in a narrower temperature range. In this case the course of the curve of change in viscosity with temperature is already independent of concentration of the polyelectrolyte, that is, RNA. Also, from these results on viscosity at different ionic strengths and various RNA concentrations at μ=0.1 it follows that the greatest possible value for rise incharacteristic viscosity, which characterizes the degree of straightening of the chain, is determined by the concentration of electrolyte, that is, the concentration of opposite ions in the medium. Thus, the maximum value for the characteristic viscosity in 6 M urea at μ= 0.6 was not more than 400 cm^3/g (65°); at μ = 0.1 , 630 cm^3/g (50°) ; at μ = 0.01, as special experiments showed, it was 1500 cm^3/g (45°). In the latter case, the especially strong rise in viscosity evidently depended on intermolecular reactions due to the sharp increase in asymmetry of the macromolecules. The lower the RNA concentration, the greater was the maximum value to specific viscosity which is attained by heating (Fig. 2). Hence, the lower the concentration of electrolyte the greater is the straightening of the chain of RNA after rupture of the hydrogen bonds between the bases.

Thus, the temperature point at which transformation to the straightened conditions begins, the width of the temperature interval and the degree of straightening attained in the transformation (according to viscosity data) will vary depending on concentration of opposite ions in the medium. All this shows that if rupture of the hydrogen bonds (change in secondary structure) occurs by the action of heat motion and urea, then the straightening of the chain as a whole (change in tertiary structure) when the hydrogen bonds have broken occurs chiefly in the bases because of electrostatic repulsion of the phosphate groups. Naturally the course of the change of secondary structure of infectious RNA (despiralization of the oriented portions of the chain) should not agree at all with the course of change of the tertiary structure (straightening of the polynucleotide as a whole) , since these are two different, through interacting processes.

In addition to the work on viscosity, we studied the sedimentation of infectious RNA in 6 M urea with μ= 0.1 at 50-55°. The sedimentation constant obtained under standard conditions and extrapolated to infinite dilution was about 10.5 Svedberg units, that is, it was 2.5 times less than the corresponding constant at 20° (Table 1). The characteristic viscosity under these conditions was 600-650 cm^3/g, that is, three to four times more than at 20° (Table 2). Hence in the solution at raised temperature the macromolecules of infectuous RNA are in a much less compact state, apparently both with respect to the compactness of the molecule as indicated by the absence of secondary structure, and in respect to spatial asymmetry.

Study of light scattering fully confirmed these conslusions, showing that a higher temperature the asymmetry actually is much higher than at 20° (Table 3, values Z) obviously, increase in asymmetry of the RNA molecule depends on unfolding and straightening of the polynucleotide chain as a result of complete breaking of the hydrogen bonds and following electrostatic repulsion of the phosphate groups of the chain. Hence, at 50-55° in a 6 M urea solution with μ= 0.1, the RNA macromolecule cannot be considered as a compact ball or a flexible statistically folded chain, but rather should be considered as a more or less straightened and rigid structure (since at this temperature it has strong electrostatic repulsion along the chain).

Calculation of the molecular weight of infectious RNA (Table 3) at raised temperature carried out from light-scattering data on the basis of the idea of the macromolecule as an extended rod showed that the molecular

TABLE 1

Sedimentation Constants of Infectious RNA

Solvent	Temperature, °C	RNA concentration, g/cm³	$S_{20,w}$ Svedberg units	$S_{20,w}$ extrapolated to infinite dilution
Phosphate buffer, pH 7.2 μ = 0.05–0.1	20	$0{,}9\cdot10^{-3}$	26,1	
		$0{,}45\cdot10^{-3}$ *	29,5	
		$0{,}18\cdot10^{-3}$	28,4	
	20	$1{,}0\cdot10^{-3}$	25,4	30—33
	20	$2{,}0\cdot10^{-3}$	21,5	
	20	$4{,}4\cdot10^{-3}$	16,0	
6 M urea, phosphate buffer, pH 7.2 ,μ = 0.1	20	$0{,}5\cdot10^{-3}$	25,0	
	20	$1{,}0\cdot10^{-3}$	24,5	
	22	$1{,}0\cdot10^{-3}$	25,8	≈27
	24	$1{,}0\cdot10^{-3}$	23,0	
	22	$1{,}2\cdot10^{-3}$	22,8	
	20	$3{,}6\cdot10^{-3}$	14,6	
	52,5	$0{,}36\cdot10^{-3}$	10,0	
	52,5	$0{,}7\cdot10^{-3}$	9,8	≈10,5
	55	$1{,}0\cdot10^{-3}$	9,4	

Note. At the given temperature, each experiment was carried out with a different preparation. In the case marked with an asterisk we used three different dilutions of the same preparation.

TABLE 2

Viscosity of Solutions of Infectious RNA

Solvent	Expt. No.	Temperature, °C	RNA concentration, g/cm³	Viscosity, η_{sp}/c	Characteristic viscosity $[\eta]$
Phosphate buffer, pH 7.2 , μ = 0.1		20	$0{,}12\cdot10^{-3}$	155	150
			$0{,}8\cdot10^{-3}$	195	
6 M urea, pH 7.2 , μ = 0.1	1	20	$0{,}11\cdot10^{-3}$	170	160
			$1{,}1\cdot10^{-3}$	180	
	2	20	$0{,}2\cdot10^{-3}$	170	165
			$1{,}0\cdot10^{-3}$	190	
	3	20	$1{,}0\cdot10^{-3}$	170	150
	4	20	$1{,}0\cdot10^{-3}$	300	240
	5	55 20	$1{,}0\cdot10^{-3}$	220	190
			$1{,}1\cdot10^{-3}$	505	
	1	50	$0{,}2\cdot10^{-3}$	630	
			$1{,}0\cdot10^{-3}$	690	
	2	60	$0{,}2\cdot10^{-3}$	610	600—650
			$1{,}0\cdot10^{-3}$	600	
	4	50	$1{,}0\cdot10^{-3}$	690	
	5	50	$1{,}0\cdot10^{-3}$	580	

Note. The constant K' in the equation $\eta_{sp}/c = [\eta] + k'[\eta]^2c$ for the solution of RNA in 6 M urea, μ=0.1 has a reproducible value of 0.9. Therefore in some cases the value of the characteristic viscosity in the corresponding experiments at 20° was calculated by the equation, diectly from the specific viscosity.

weight in general kept the same value as at room temperature ($1.5\cdot10^6$). Hence unfolding of the nucleotide chain is not accompanied by a fall in molecular weight. In other words, destruction of the secondary and tertiary structures by heating is not accompanied by destruction of the primary structure of infectious RNA. All these results show that the primary structure of infectious RNA is a single continuous polynucleotide chain with molecular weight $2\cdot10^6$.

TABLE 3

Light Scattering of Solutions of Infectious RNA in 6 M Urea, $\mu = 0.1$

RNA concentration, g/cm³	Temperature, °C	R_{90}	$Z = \frac{R_V}{R_H}$	P_{90}^{-1}	M	L, Å
$1 \cdot 10^{-3}$	20,2	$12,7 \cdot 10^{-5}$	1,9	1,75	$1,7 \cdot 10^6$	2200
	47,5	$5,2 \cdot 10^{-5}$	2,5	≈3,5	$\approx 1,5 \cdot 10^6$	—
	20,5	$9,7 \cdot 10^{-5}$	1,9	1,75	$1,3 \cdot 10^6$	2200
$0,4 \cdot 10^{-3}$	20,6	$9,6 \cdot 10^{-5}$	1,7	1,55	$2,9 \cdot 10^6$	1600
	53,5	$3,5 \cdot 10^{-5}$	2,1	2,15	$1,5 \cdot 10^6$	—
	21,3	$8,1 \cdot 10^{-5}$	1,7	1,55	$2,4 \cdot 10^6$	1600

Note. In both these experiments the solution was first measured at room temperature (upper line for each experiment), then it was heated to 47-53° (middle line), after which it was cooled and again measured (lower line). Data are not given for zero RNA concentration since the possible correction is much less than the variation in the values given for the different preparations. Measurement was carried out at $\lambda_0 = 5460$ A. P_{90}^{-1} and L/λ' were found from the value for Z in the corresponding graph [12] starting from the model rigid rod. The density of RNA was taken everywhere as d=1.67 g/cm³ [3,4], dn/dc = 0.19, n for 6 M urea is 1.384.

The data of the previous work on the study of infectious RNA at higher temperature [1] showed that a complete primary structure of virus RNA and the absence of breaks in the chain within the macromolecule in solution are evidently necessary conditions for the biological activity, infectiousness.

Change in Properties of RNA when the Heated Solution Is Cooled

A striking feature of infectious RNA is the complete reversibility of its physicochemical properties when the heated solution is cooled.

As measurements of optical rotation showed during cooling of solutions of infectious RNA $[\alpha]_D$ rises with fall in temperature (as in the case of cooling and RNA solution in phosphate buffer which had been heated to 80° or on cooling a solution of RNA in 6 M urea which had been heated to 60°). We then find complete reversion of the optical rotation. Analogous results were obtained for the ultraviolet absorption of RNA solutions.

This permits us to believe in the restoration of the secondary structure of infectious RNA which had been lost when the solution was heated.

Similar observations were made with regard to the tertiary structure. The values for the specific viscosity of the infectious RNA solutions after their heating and later cooling were the same as in the preparation before heating. Complete reversibility was also shown for the sedimentation.

One of the preparations of infectious RNA in 6 M urea with $\mu = 0.1$ was submitted to a sedimentation study at 20° in three variants: without preliminary heating of the solution; after 15 min heating of the solution at 50° with later rapid cooling to 20°; after heating the solution three times at 50° (for 5 min) with rapid cooling to 20° after each heating. All three variants gave the same sedimentation picture, with one narrow peak (Fig. 3): $S_{20,w}$ = 22.8, 23.4, 22.4 Svedberg units respectively, that is, they were practically the same.

Reversion was observed in the value of the molecular weight and asymmetry Z calculated from light scattering (Table 3). Thus, the combination of the data indicates the ability of high molecular weight infectious RNA to regain the tertiary structure also, that is, to become folded and coiled into a definite rod-like compact macromolecule, monodispersed as to dimensions and form.

Special experiments to test the infectiousness of the RNA preparations during the study showed that after the solutions had been heated, their infectiousness did not change. Hence, the reversible changes of secondary and tertiary RNA structure in solution do not affect the biological activity of the preaparations studied.

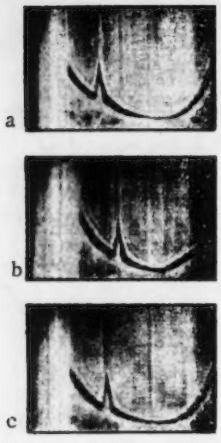

Fig. 3. Sedimentation diagram of infectious RNA in 6 M urea solution, pH 7.2, μ = 0.1, before and after heating the solution at 50°. RNA concentration 0.1%; 20°; 40,000 rpm; a) before heating; b) after 15 min heating; c) after heating three times, 5 min each time.

DISCUSSION

Turning to a consideration of these results we must remember that the RNA chain within the virus particles is a single-chain extended spiral with a pitch of 23 A as it would fit into the protein fiber portion [14]. Then, obviously, the separate portions and links of the polynucleotide chain cannot interact with each other by hydrogen bonds or by van der Waals or ionic forces. Hence, deproteinization of the virus, which fully liberates the RNA from its previous protein surroundings and makes possible the interaction of the separate links and portions of the chain with each other depends entirely on the destruction of the previous "native" configuration of the polynucleotide chain. This chain is coiled in water solution into a compact, rod-like molecule. Just this macromolecule with its secondarily formed secondary and tertiary structures has been studied as the "macromolecule of infectious RNA from tobacco mosaic virus".

Hence, the secondary and tertiary structures of infectious RNA in solution are in no way specific for the native structure. They are simply the result of a nonspecific coiling with spiral formation of separate portions of the chain where the succession of nucleotides permits this to occur. As a result, a compact rod is formed with a definite percentage of spiral formation and a definite degree of spatial asymmetry and form, determined by the possibilities which are presented for coiling and spiral formation by the succession of nucleotides themselves and by the equilibrium forces which are established within the macromolecule and between the polynucleotide and the solvent.

These ideas permit understanding of the complete reversibility of all the changes of secondary and tertiary structures of infectious RNA in solution.

SUMMARY

The highly polymerized RNA obtained from tobacco mosaic virus by phenol deproteinization and which has infectious activity was studied from the standpoint of its macromolecular structure in solution. We made measurements of optical activity, viscosity, sedimentation, and light scattering at different temperatures.

We showed that in solution at room temperature the RNA macromolecules are compact, rigid rods with molecular weight about 2 million, and a ratio of length to diameter of 60-80, with a secondary structure (apparently oriented spiral portions within the macromolecule, held by hydrogen bonds), and tertiary structures (which fold and coil the entire chain into a compact rod). The presence of 6 M urea does not destroy this structure, though it results in a looser stage of the macromolecule and decreases the quantity of oriented (spiral) portions in it because of partial rupture of the hydrogen bonds.

When the solution of infectious RNA is heated there is destruction of the secondary structure (despirilization of portions of the chain), and unfolding (straightening) of the polynucleotide chain with transformation from a compact to a looser extended state. The presence of 6 M urea permits the destruction of the former secondary and tertiary structures more easily by shifting the transformation temperature 20° lower. We have shown that the destruction of the secondary structure (despirilization of portions of the chain) occurs because of rupture of the hydrogen bonds, while the change in tertiary structure depends chiefly on strengthening of the electrostatic repulsion of the phosphate groups in the chain, that is, these two processes, though interdependent, are different in nature.

Under conditions of raised temperature with full loss of secondary structure and maximum unfolding of the chain, the macromolecule of infectious RNA is characterized by the same high molecular weight as at room temperature. We must conclude that the primary structure of infectious RNA is a single continuous polynucleotide chain with molecular weight about 2 million.

When the heated solutions are cooled all the physicochemical changes revert to the original values, that is, there is complete restoration of the secondary and tertiary structures with formation of the former compact rods, monodisperse with regard to dimensions and form.

We conclude that the secondary and tertiary structures of the RNA macromolecule in solution are not specific to the native structure but arise secondarily after deproteinization.

We thank Prof. A. N. Belozerskii for his constant attention and help, and also V. G. Aldoshin for carrying out experiments on light scattering.

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* See English translation.

** Original Russian pagination. See C.B. Translation.

SCIENTIFIC INFORMATION

ALL-UNION CONFERENCE ON THE BIOCHEMISTRY OF GRAIN AND OF BREAD BAKING

The projects of the XXI Congress of theCommunist Party of the Soviet Union and the Seven Year Plan for development of the national economy of the USSR require a great increase in production of grain in the USSR as a basis for all agricultural production and an improvement in the quality of food products; this presents to Soviet science, and especially to grain and baking biochemists, a series of important problems.

For the solution of such important questions as the discovery of types of wheat with high protein content, working out methods of drying and long keeping of the grain, perfecting technological processes under conditions of automatic conveyer belts in elevators, mills, flour and bread factories we must have close accord between the technical workers and the biochemical investigators. The study of the biochemical processes which occur in the grain during its harvesting, keeping, treatment, and also in the making of bread will give a true answer to these problems.

It was with these problems that the All-Union Conference on the Biochemistry of Grain and Bread Making was concerned; it was organized by the A. N. Bakh Institute of Biochemistry and the All-Union Institute of Cereals together with the Ministry of Higher Education of the USSR, the State Committee of Soviet Ministry for Bread Making, and the State Planning Committee of the RSFSR. The Conference met April 9-11, 1959 in Moscow in the Division of Biological Sciences of the Academy of Sciences of the USSR. Not only scientific workers took part in the Conference as members of various research institutes and institutions of higher learning of the nation, but also practical w orkers in bakeries, mills, the State Commission for testing agricultural products, and advisers and members of the Ministry of Bakery Products.

In a report on the biochemical problems in the field of cereal economy and the flour and baking industries, the president of the organizing committee, Academician A. I. Oparin remarked that the theme of the Conference centered around the following important problems: cereal proteins, their properties, method for investigation and significance in estimating the quality of the grain, flour, and bread; enzymes of grain and flour, their properties and role in various stages of drying, keeping and treating the grain, and also in the processes of preparing bread; the action of irradiation of grain and the products of its treatment; new methods for estimating the quality of the raw materials and the control of technological processes.

In opening the Conference, the Academician Secretary of the Division of Biological Science, Academy of Sciences, USSR, Corresponding Member N. M. Sisakyan said that the purpose of the conference was not only to report work which had been performed, exchange experiments, and find further ways of solving scientific and practical problems, but also to consider the initiation of systematic work on the coordination of research on the biochemistry of grains, flour, and bread, in which the Division of Biological Sciences, USSR, (Curator, A. N. Bakh Institute of Biochemistry) should assume the role of initiator.

The problems of biochemistry of grains and baking in connection with the Seven Year Plan for developing the national economy of the USSR were the subject of the address by Professor V. L. Kretovich (A. N. Bakh Institute of Biochemistry). Starting from the resolutions of the XXI Congress of the Communist Party, he emphasized in his speech the need for close agreement in the work of selection and choosing new kinds of wheat and biochemical investigation, chiefly of grain proteins and enzymes which are important factors in determining the baking quality of wheat, rye, and other crops. In connection with the growth of cereal production in the USSR, an urgent problem

is the organization of annual surveys of the level of cereal culture with respect to the quality. In the address he remarked on the value of scientific research for working out a regime of drying the grain, especially in the eastern part of the country, and also for developing a realistic plan for the technical processes in flour mills and the baking industry, particularly in connection with the introduction of complex mechanization and automatic production processes. V. L. Kretovich emphasized the great value of biochemical investigation for developing methods of determining the quality of grains and flour and improving the taste and aroma of bread, and also methods for enriching the bread with proteins, vitamins, and minerals. In his address he remarked on the need for introducing new research methods into the studies : chromatography, electrophoresis , use of labeled atoms, etc.

A series of addresses at the Conference were devoted to the question of technical evaluation of different types of wheat grown in the USSR. A basic indicator of the quality of wheat is the content of gluten and its physicochemical state. As M. M. Samsonov (State Commision for Testing Agricultural Products) stated, our country has the most favorable natural and economic conditions for further increase in cereal production and for growing high quality hard wheat. As a result of the general results obtained over a great many years on a large amount of material, it has been established that there is a relation between gluten content and type of wheat, place of production, and agricultural techniques. The baking properties of a number of types of wheat raised in our country were discussed in the speech of I. N. Maslov (Institute of Baking Industry). The address of T. B. Darkanbaev (Kazakh University) was devoted to the results of a study of the Biochemistry of Kazakhstan wheat. The results permitted a wide comparison of the biochemical characteristics of Kazakhstan wheat with respect to content and properties of proteins, behavior, of gluten, and sugar and starch content.

On the basis of biochemical characterization, the technical quality of new types of wheat was considered and methods for their rational use in the food industry were discussed. The results of a study of biochemical and technical quality of the wheats of Kulund were presented in the report of K. P. Volgina (West Siberian Branch, Academy of Sciences, USSR).

At the present time a comprehensive study is under way on the problems of use of ionizing radiation to combat blights and microorganisms which cause damage to cereals. The question of the action of radiation on grain was discussed by N. I. Sosedov, A. B. Vakar, and O. P. Pod"yapol'skaya (Institute of Cereals and the Products of Their Treatment) and by A. D. Chmyr (Odessa Technological Institute). These addresses gave data on the action of x-rays and γ-rays in different doeses on proteins, fats, and carbohydrate complexes of wheat and corn with differing moisture in the grain and varying doses of radiation.

One of the most important problems of the biochemistry of grain is the study of proteins and enzymes of wheat, rye and other grain crops as the most important factor in determining their merits for baking and food. These investigatiolns are also very valuable for selectors who have the problem of evolving new types of wheat with high protein content. At the conference a large number of addresses were devoted to a study of the structure and properties of wheat protein and gluten. In the address of V. M. Kolesov (Tomsk Medical Institute) were given data on the structure of cereal proteins (mostly of wheat) and their comparative characteristics of chemical and physicochemical properties; M. S. Reznichenko (Leningrad Institute of Soviet Trade) spoke of the results of a study of the terminal groups of the gliadin molecule; Zh. V. Uspenskaya (A. N. Bakh Institute of Biochemistry) reported the results of a study of methods of biosynthesis of phenylalanine and tyrosine in growing wheat spikes. The investigation showed that in spikes there is intensive formation of phenylalanine and tyrosine from phenylpyruvic acid. The results of a study of amino acid composition of far eastern varieties of soybeans were given by I. F. Belikov, E. Ya. Nedel'ko, and P. Tyuleneva (Far Eastern Branch of the Academy of Sciences, USSR). The physicochemical and enzymatic properties of the reserve protein of soybeans, glycinine, were described by T. L. Smirnova (A. N. Bakh Institute of Biochemistry), using new physical and chemical methods to obtain and study the protein. The author showed that glycinine from soy cotyledons is a homogeneous protein and consists of identical submolecules with molecular weight of 20,000 . These submolecules form a hexamer whose molecule in turn can give further reversible association with the formation of a macromolecule, an oligomer. The studied glycinine preparations had β-amylase, lipoxidase, and urease activity. V. F. Golenkov (Institute of Cereals and Products of Their Treatment) gave data on some conditions for formation of rye gluten and its physical and chemical properties. The changes in gluten under different conditions of ripening of wheat (in cuttings from different harvests, in keeping the grain) were reported in the address of S. I. Akivis, A. B. Vakar, I. E. Mambish, and N. I. Sosedov (Institute of Cereals and the Products of Their Treatment). It was established that when the wheat lay in the field in the second phase of ripeness, there was an improvement in the quality of the gluten. This process continued when the grain was kept in the warehouse. N. V. Romenskii and co-workers (Odessa Technological Institute)

reported the results of work on studying changes in wheat proteins in connection with some conditions of cultivation and technical processes of the flour milling industry. The work continued for several years with wheat raised in the southern Ukraine in the region of Eurygaster infestation. N. V. Romenskii suggested a method of improving the baking quality of grain damaged by blight by the treating it with chlorine water.

Much consideration was given at the Conference to the question of drying the grain. In connection with the growth of grain production in the eastern part of the country, where there is increased moisture in the grain during the harvesting period, one of the most important problems is to work out a biochemical basis for a plan of drying the grain and a time for production.

E. G. Onishchenko (Moscow Technological Institute for the Food Industry) spoke of the results of work on the effect of gases from various types of coal on the physical and chemical properties of wheat gluten when the grain was dried in a grain fryer of the shaft type. I. I. Lenarskii (Uzhgorod University) indicated in his address that the use of a regime of drying the grain and methods of using heat to affect the enzymes of the grain did not always consider the laws of heat denaturation of the proteins. This involved either decreasing the effectiveness of the process or worsening the quality of the grain. However, experiments showed that if the heat regime of treatment was set up on the basis of the kinetics of heat denaturation of the grain proteins, it was possible to inactivate the proteolytic enzymes of the grain, which are injured by the Eurygaster without actually changing the properties of the gluten protein complex. E. I. Medvedeva (Odessa Technological Institute) reported the results of experiments to explain the occurrence of physicochemical changes of gliadin when heated. At temperatures of 60 and 70° in gliadin with moisture content of 18 and 20% there were changes in the structure of the molecules, which, however, were not related to their destruction. Yu. S. Rall' (Timiryazev Academy) reported a study of the effect of heating corn kernels on their germination and some biochemical properties which was of great value in working out a scheme for drying the corn kernels.

At the present time there is very important work dealing with the development of new methods for increasing the taste and nutritive properties of wheat products. R. R. Tokareva (Institute of the Baking Indsutry) reported the use of enzyme preparations to improve the quality of wheat bread by adding to the dough, purified concentrated enzyme preparations isolated from cultures of Aspergillus oryzae.

The paper of T. L. Auerman, F. I. Zaglodina, and N. I. Proskuryakov (Moscow University) reported the action of ascorbic acid on the protein-proteinase complex of wheat flour and prospects for its use in the baking industry. N. V. Leonovich (Brewing Institute) told of studying the enzymes of malt sprouts and their use in brewing I. Sh. Shklovskii (Odessa Technological Institute) gave a paper on the results of experiments on the effect of oxidizing and reducing agents on the proteinases and gluten of wheat. He showed that reducing agents, which cause "weakening" of the gluten, act directly on the gluten and not on the proteases of the flour.

There was practical value in the investigations of enzymes (catalase, amylase, lipase) from the kernels of hybrid corn carried out by V. A. Yakobenko and L. P. Torzhinskaya (Odessa Technological Institute). Their experiments showed a connection between enzymatic activity and phases of ripening, drying, keeping and the economic value of hybrid corn.

In order to solve the problem of further increasing the quality of production of flour mills and the baking industry we must expand the complex procedures which affect the biochemical properties of flour. A study of the biochemical changes which occur in wheat grains when new technical methods of treating the grains are used was described by N. P. Koz'mina and N. N. Zotova. N. P. Koz'mina (Institute of Cereals and Products of Their Treatment) reported that studies in recent years have shown the principle methods which are used in treating the grain lead to great changes in the biochemical properties of the flour. Thus, on the basis of studies of the micro- and submicrostructures of the endosperm of cereals processes have been found for treating flour with an increase in protein contant, and the construction of machines which has been worked out for cutting up the grain (laureate of the Kossuth Prize, Engineer P. Raikai, Hungary) permits us to consider regulating enzyme activity by removing the embryo portion of the seed. Experimental studies under laboratory conditions confirm the possibility of lowering the activity of amylolytic enzymes and thus improving the quality of the bread from sprouting grain. N. N. Zotova (Moscow Technological Institute for the Food Industry) reported the results of studying the biochemical processes which occur in the wheat grain in conditioning. It was established that during hot and cold conditioning with 16.5% moisture in the grain the yield of crude gluten increased, its physicochemical properties, were changed, and there was also a change in free amino acid content which could improve the baking qualities of the flour.

In the field of the biochemical basis of baking the most important questions are the study of biochemical processes under conditions of the automatic moving belt and the development of methods, indexes and norms which reflect actual baking with good flour.

In the address of V. V. Shcherbatenko (Institute of the Baking Industry) data were given on the study of biochemical processes which occur in the new automatic belt method of preparing dough. K. N. Chizhova (the same Institute) gave the results of a study of the changes which occur during dough preparation in the protein complexes of flour (gluten was studied as the whole complex, without separating it into fractions). The study gave the total changes in gluten protein during bread manufacture under production conditions and the changes in protein in model experiments where gluten extracted from flour was submitted to the action of various factors. The results of a study of the characteristics of protein-proteinase complexes of wheat flour with short-cleaving gluten and a method for improving the bread baked from it was reported by E. I. Vedernikova and co-workers (Ukrainian Research Institute for the Food Industry).

Numerous problems were set before the biochemists in connection with the need for fundamental improvements in technical control of cereals, flour, and bread. The success of this work depends to a considerable degree on the use in research work of new physicochemical and biological methods of investigation.

In the concluding session of the Conference there were a number of papers on the methods used for determining the quality of grain, flour, and dough. L. N. Lyubarskii, I. E. Mambish, and S. S. Suvorov (Institute of Cereals and Products of Their Treatment) reported a new method of determining the moisture of grains by a Russian Electrohumidity meter. The method guarantees reproducible results within ± 0.05% V. L. Kretovich and A. P. Prokhorova (A. N. Bakh Institute of Biochemistry and Central Laboratory of GUGMP) described a new method of determining the odor of grains. The method is based on separation of volatile substances from the grain, those which give it some specific odor. I. E. Mambish (Institute of Cereals and Products of Their Treatment) reported the determination of the whiteness of flour on the basis of an objective method and on a new construction of laboratory apparatus for estimating the quality of the flour by its whiteness. L. Ya. Auérman (Moscow Technological Institute for Food Products) presented a detailed comparison of various method of determining the physical properties of gluten and dough and described the development in his laboratory of determining by an automatic penetrometer of type "Labor-365" the physical properties of gluten and dough which characterize the "strength" of wheat flour. K. P. Petrov (Kiev Technological Institute for the Food Industry) reported on a refractometric method for determining the diastase activity of flour, malt, and other plant products.

In concluding the sessions of the Conference, N. P. Koz'min gave information on the establishement of an International Society on Cereal Chemistry.

The resolutions adopted by the Conference reflect the basic problems which are presented to cereal and bakery biochemists in connection with the decisions of the XXI Congress of the Communist Party. Resolutions were adopted on the coordination of scientific work in this field, and the plan of the Division of Biological Sciences of the Academy of Sciences, USSR, to entrust the implementation of this plan to the A. N. Bakh Institute of Biochemestry of the Academy of Sciences, USSR, was approved. It was also decided to convene conferences on the biochemistry of cereals and baking systematically to discuss definite themes (questions connected with the moisture of grains, flour and bread and method for its determination). It was also considered important to publish the proceedings of the Conference in the near future.

The members of the Conference were able to become acquainted with new biochemical methods for investigation in the laboratories of the A. N. Bakh Institute of Biochemistry, the All-Union Institute of Cereals, and also the Food Institute and the Institute of the Baking Industry.

Z. G. Evstigneeva

REVIEW

D. Nashkov and R. Shipolini. "Paper Electrophoresis".

198 pages , Izdatelstvo "Meditsina i Fizkultura", Sofia, 1957

The book is devoted to the principles and method of paper electrophoresis and its use in clinical and medical practice.

Electrophoresis as a method of investigation has become more and more widely introduced into biochemical practice. A discussions of the theory of electrophoresis and the possibilities of its use have been the subject of a number of works. In the book under review there is a detailed consideration of one of the most important modifications of electrophoresis, paper electrophoresis. Starting from a theoretical discussion, the authors have considered in detail all the possibilities of the use of this method and have described the different apparatus used in paper electrophoresis.

The book of D. Nashkov and R. Shipolini provides an introduction to one of the original modern methods of electrophoresis, that of Arne Tiselius; it consists of an introduction and four chapters, each of which contains a number of divisions. At the end of each division there is a detailed bibliography. However, in the list of literature there is not given the full name of the work cited, which makes the use of the references somewhat difficult. The book contains a large number of figures, diagrams, tables and plans which makes understanding of the material easier.

The introduction briefly explains the advantages of the paper electrophoresis method compared to other forms of electrophoresis. The first chapter, "Theoretical Basis" briefly describes the history of the development of electrophoresis and gives the theoretical basis for the migration of particles in an electric field in free solution and on paper. In particular it describes the conditions for carrying out paper electrophoresis and the factors which affect the electrophoretic mobility of particles. It considers in detail the question of the effect of evaporation of the liquid from the surface of the paper as the substance migrates on the paper and gives the theoretical basis for observing this effect and also the relation of mobility of substances to time, current strength and voltage, temperature, pH, and ionic strength of buffer solutions.

In the second chapter, "Methods" it considers the principle types of apparatus for paper electrophoresis and describes the basic form of the apparatus used. The authors divide the apparatus into three main groups. The first group consists of apparatus in which evaporation is completely prevented. This is done in various ways: the paper is put into chlorobenzene, carbon tetrachloride, toluene, and other organic solvents (using outside cooling), or is enclosed in paraffin layers between plates of glass or polyethylene sheets, using water in the cooling system. The second group includes apparatus with a moist chamber. The authors describe various plans for apparatus of this type, with horizontal or nonhorizontal arrangement of the paper strips and with cooling of the working chamber. The third group contains apparatus with continous evaporation; some have cooling systems which are placed under the strip of paper and maintain the desired temperature. There are also detailed descriptions of the techniques of investigation including filling the bath with buffer, fastening the strip, introducing the sample (volume of sample deposited), carrying out the electrophoresis, and fixing the electrophoretogram. The authors give a theoretical analysis of methods of continuous electrophoresis, the mobility of particles on the paper under the influence of a definite applied electrical field with continuous supply of the mixture to be separated. In this section they consider questions of the distribution of the electrical field on the strip of paper depending on the buffer solution used, the pH, the preparation of the mixture to be separated , the possibility of using two-phase electrophoresis,

and electrochromatography, and they give orienting characteristics for various types of paper and buffer solutions. They consider in detail the question of using dyes to color the electrophoretogram, the relation of the color to conditions of its application and the effect on it of various factors: time of dyeing, duration of washing. They describe the various processes for coloring the protein fractions on the electrophoretogram with a number of dyes, and the quantitative determination of these fractions. They describe processes for quantitative determination of the fractions by densitometric determination in visible or ultraviolet light, and different methods of elution. Then they describe apparatus for densitometry and elution. At the end of the chapter they discus the question of the accuracy of the electrophoretic method for various proteins and the limits of variation. The authors show that the method is entirely usable for quantitative determination of proteins when the average values are used from three different electrophoretic determinations.

In the third chapter, "Use of Paper Electrophoresis in Laboratory Practice", the question of investigation of human and animal blood serum proteins is discussed and also the electrophoresis of bile, and snake and bee venoms; there is detailed discussion of electrophoresis of structural proteins, especially muscle proteins; the electrophoresis of lipoproteins and method for staining them are described, and also method of determining glycoproteins and a number of enzymes of animal origin. They consider methods of electrophoretic separation of amino acids, nucleic acids, and the products of their breakdown; the separation and determination of some vitamins and organic acids. In the last sections of the chapter are described electrophoretic investigations of monosaccharides and their methyl esters, disaccharides and polysaccharides, polyvalent alcohols and phenols, and also the separation of steroids and antibiotics.

In the fourth chapter, "Electrophoresis and Its Use in Clinical Practice", they consider the case of the use of the electrophoretic picture of protein fractions, chiefly of the blood serum, in various diseases.

All these facts indicate that the questions considered in this book are discussed throughly and from many viewpoints. However, it should be mentioned that the work would be still more valuable if it contained examples of the use of paper electrophoresis in investigations on the biochemistry of plants and in different branches of technical biochemistry.

In the book as a whole, D. Nashkov and R. Shipolini give a complete monograph on paper electrophoresis which will certainly be widely used among specialists in their research and practical work.

T. Florenskaya

NOTICES OF MEETINGS

V INTERNATIONAL BIOCHEMICAL CONGRESS

In conformity with the decision of the General Assembly of the International Biochemical Union, the Academy of Sciences of the USSR will organize in Moscow, August 10-16, 1961, the V International Biochemical Congress. The basic work of organization and conducting of the Congress falls to the All-Union Biochemical Society.

The Presidium of the Academy of Sciences of the USSR has named Academician A. I. Oparin as President of the Congress, and Corresponding Member of the Academy of Sciences N. M. Sisakyan as General Secretary of the Congress. The scientific comittee, which will include 88 biochemists of various specialties, will be headed by Academician V. A. Éngel'gardt.

At the present time much preliminary work is being carried on. The determination of the basic scientific problems on which the attention of the Congress will be centered are being formulated under the head of eight symposia and the groups of questions around which each of these will center are being defined, and sections for short experimental communications by member of the Congress are being planned. These measures have been widely discussed in meetings of the All-Union Biochemical Society and are also being approved by a large number of leading foreign biochemists. All the preliminary work has been published in the first circular of the Congress in Russian, English, and French. At the present time the circular has been sent to the national biochemical societies and to many biochemical institutes and university departaments, and also to individual biochemists in all countries of the world.

In distinction to previous biochemical congresses, the attention of the participants of the V International Congress will be devoted to the most pressing and promising problems of comtemporary biochemistry, and first to the problem of "structure and function". The lectures in the Plenary Sessions of the Congress and the chief symposia will be organized from this point of view, and will consider the following problems:

1. Biopolymers, their molecular structure and biochemical activity (President, M. Perutz, England; organizer V. A. Engel'gardt). The trimeric structure of the most important biopolymers, proteins and nucleic acids, the interconnections of molecular structure and biochemical properties of enzymes, hormones, viruses; enzymatic syntheses of biopolymers and mechanism of their reactions; molecular structure of contractile proteins; structural chemistry of antigens and antibodies; physical and chemical methods for study of biopolymers.

2. Functional biochemistry of cell structures (President, B. Lundberg, Sweden; organizar, N. M. Sisakyan). Localization of biochemical function in the cell; change of enzymatic properties during development. Reactions of various intracellular structures in metabolism. Questions of comparative biochemistry of intracellular structures.

3. Evolutionary biochemistry (President, S. Ochoa, USA; organizer, A. I. Oparin). Different methods of carbohydrate metabolism in relation to evolutionary development. Evolution of anaerobic metabolism. Origin and evolution of aerobiosis. Origin and evolution of autotrophy, chemosynthesis and photosynthesis. Evolution of nitrogen metabolism.

4. General mechanisms of action and specific inhibition of enzymes (President P. Desnuelle, France; organizer A. E. Braunshtein). Explanation of the chemistry of enzymatic reactions on the molecular level. Questions of the structure of the catalytically active centers of enzyme proteins; their blocking by specific reagents; interpretation of enzymatic reactions on the basis of modern structural theory and transformations of organic molecules. Mechanism of reactions between enzymes and structurally analogous substrates and coenzymes (antimetabolites).

5. Phosphorylative and nonphosphorylative oxidations (President, E. Slater, Holland; organizer, S. E. Severin). General principles of mechanism of phosphorylative and nonphosphorylative paths of oxidation; combination reactions and their relation to structural mitochondria. Functions of oxidation in energy metabolism (accumulation of energy in usable form and heat production) and in plastic metabolism.

6. Mechanism of photosynthesis (President, H. Tamiya, Japan; organizer, A. A. Krasnovskii). Nature of the primary photochemical process. Photochemistry and spectroscopy of the pigments. Enzymatic stages of photosynthesis photosynthetic phosphorylation. Biosynthesis of pigments and their condition in the organism. Products of photosynthesis, their connection with respiration and nitrogen metabolism. Bacterial photosynthesis.

7. Basic mechanisms and paths of biosynthesis. The content of the symposium will be determined in the final program.

8. Biochemical basis of the techonology of food products (President, E. Pianovskii, Poland; organizer, V. L. Kretovich). Biochemical principles of selection of agricultural plants. Biochemical basis for technology of raw materials of plant and animal origin. Biochemical basis for technology of fermentation products.

It should be remarked that this is the first practical international biochemical congress to be organized in symposia to deal with the most pressing problems of technical biochemistry which have been so widely developed in our country.

At the present time there has been a great deal of work in setting up the detailed program of the symposia and and the invitations for the addresses. According to the plan mentioned, each symposium will have four sessions lasting 3 hours each. This will permit hearing at each session of one or two main speeches and a number of fixed papers and also an exchange of free discussion of opinions on interesting questions. The addresses presented in the symposia will be printed and distributed to the members of the Congress beforehand.

The experimental communications by the members of the Congress can be submitted to 27 sections devoted to various biochemical questions: chemistry of biologically important compounds, biochemistry of proteins and amino acids, biochemistry of nucleic acids and nucleoproteins, biochemistry of viruses, enzymology, comparative biochemistry. biochemistry of antibiotics, industrial biochemistry, cyto-and histochemistry, biochemistry of muscle, biochemistry of the nervous system, vitamins and nutrition, biochemistry of hormones, biochemistry of microorganisms, biochemistry of plants, medical biochemistry, biochemistry of carbohydrates, biochemical pharmacology, biochemical genetics, immunochemistry, biochemistry of malignament neoplasms, biochemistry of photosynthesis, respiration and oxidation, action of radiation on biochemical processes, biogeochemistry, new apparatus and methods in biochemistry, miscellaneous. The reports of these papers will be published later.

At the time of the Congress there will be organized a display of scientific literature, biochemical preparations and reagents, and also equipment and apparatus used in biochemical investigations.

The organization and conduct of the V International Biochemical Congress in Moscow will lay a great responsibility on the biochemists of our nation. We cannot doubt that they will eargly undertake this task, so important for the further development of biochemical science.

G. A. Deborin

THE ALL-UNION BIOCHEMICAL SOCIETY

June 8, 1959 in Minsk there was a meeting of the Central Council of the All-Union Biochemical Society where the address of S. E. Severin was heard and considered: "The Plans and Work of the Affiliates and Sections of the Society in the Light of the Resolutions of the XXI Congress of the Communist Party of the USSR". In this speech of the member of the Central Council it was shown that the basic problem for the plan of work of the Central Council is to set up a comprehensive analysis of the research in biochemistry in the branches and sections of the society by carrying on consultative work and visits by the members of the Central Council to the sections, considering concrete relations of scientific research with practical problems which come before the

scientific and practical groups, correcting and confirming the plans of work of the affiliates and sections of the society, and introducing measures for the advance of biochemical knowledge.

In this connection the meeting confirmed the suggested plans of work for the Azerbaidzhan, Leningrad, Moscow, and Estonian Branches. All the other affiliates and sections were advised to work out a plan by July 15, 1959. The proposed shift in the symposium on the problem "Proteins and Nucleic Acids", from October, 1959 (Moscow Biochemical Society) to November 1959 was approved. It was suggested that the branches and sections of the Society set up plans for symposia and conferences on the coordination of theoretical and practical investigations and the inclusion of agricultural institutions in the work of the symposia. It was decided to receive these plans at the Central Council on September 15 of this year. The Presidium of the Central Council was instructed to present to the Press of the Academy of Sciences of the USSR a plan for a publication by the Society , with the view of a periodical of survey and monograph type, the "Progress of Biological Chemistry".

It was decided in 1960 to hold an All-Union Conference on the question of the most rational form of training biochemical groups in a number of universities, agricultural an medical institutions; to ask the Presidium of the Central Council to appoint a committee to prepare for this conference which should include workers from the Higher Schools; to permit the Presidium to recomend editorial assistants for the biochemical journals (Biokhimiya, Voprosy Meditsinskoi Khimii, Byulleten' Éksperimental'noi Biologii i Meditsiny, Ukrainskii Biokhimicheskii Zhurnal), to establish closer contact with the authors by attractin members of the Society to review work and helping the authors from the outside.

In discussing the addresses of V. A. Leonov, A. S. Vecher, T. N. Godnev, M. F. Mereshinskii, and S. A. Pozdnyak on the development of biochemistry in the Belorussian SSR, the emembers of the Central Council noted that in the Belorussian SSR biochemistry had developed in several directions : the biochemistry of plants, microbes, animal and human biochemistry, clinical and technical biochemistry. However, along with the positive results in the work of the Belorussian biochemists there were a number of deficiencies which required correction in the near future, namely: lack of study of the basic biochemical processes which occur in cells and intracellular structures; little study of the mechanism of different biologically active substances, etc. Suggestions were made as to the organization of methodological assistance to the Belorussian biochemists by the Central Council of the All-Union Biochemical Society and the larger biochemical institutes of the USSR (Institute of Biochemistry of the Academy of Sciences of the USSR, Biochemical Institute of the Academy of Sciences of the Ukranian SSR, Institute of Medical and Biological Chemistry, Academy of Medical Sciences, USSR, and other institutes where there are now biochemical laboratories). Such help should include places for performing work in the institutes mentioned, introduction of modern methods in the biochemical laboratory of the Belorussian SSR, discussion in joint conferences of the results of scientific investigations, etc. The biochemists of the Belorussian SSR should devote serious attention to preparations for participation in the V International Biochemical Congress. From these ideas, the Plenum of the Central Council of the All-Union Biochemical Society resolved: to approve the direction of work in biochemistry in the Belorussian SSR; to establish closer contact in carrying out investigations among biochemists, physicists, chemists, biologists, and physicians. Biochemical investigations carried out in Belorussia should, in addition to their general theoretical significance, answer the needs of industry, agriculture, and maintainance of health in the republic.

E. V. Budnitskaya

ERRATA

Biochemistry Vol. 23, No. 5 September-October 1958

Page	Line	Reads	Should read
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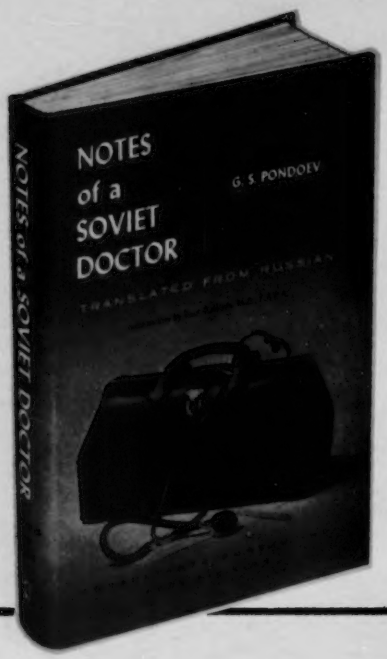

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